USING HETEROLOGOUS SYNAPSE SYSTEMS TO STUDY THE IMPACT OF POSTSYNAPTIC MOLECULES ON PRESYNAPTIC STRENGTHENING AT EXCITATORY SYNAPSES

by

Kamesh Krishnamurthy

Submitted to the Graduate Faculty of

the University Honors College in partial fulfillment

of the requirements for the degree of

Bachelor of Philosophy

University of Pittsburgh

2010

UNIVERSITY OF PITTSBURGH

UNIVERSITY HONORS COLLEGE

This thesis was presented

by

Kamesh Krishnamurthy

It was defended on

April 5, 2010

and approved by

Henry C Zeringue, Ph. D., Dept. of Bioengineering

Sanjeev Shroff, Ph. D., Dept. of Bioengineering

Jon Johnson, Ph. D., Dept. of Neuroscience

Gong Chen, Ph. D., Dept. of Biology, Penn State University

Thesis Advisor: Henry C Zeringue, Ph. D., Dept. of Bioengineering

USING HETEROLOGOUS SYNAPSE SYSTEMS TO STUDY THE IMPACT OF POSTSYNAPTIC MOLECULES ON PRESYNAPTIC STRENGTHENING AT EXCITATORY SYNAPSES

Kamesh Krishnamurthy, B.Phil.

University of Pittsburgh, 2010

The field of neurobiology focuses on the development, maintenance, and function of the nervous system. Of particular interest is the formation of synapses, the junctions which allow for transmission and control of information between neurons. Synapse formation can be broken into two general processes: structural formation and activity-dependent validation. Structural formation requires transmembrane adhesion proteins that connect the two sides of the synapse. This newly- formed connection is then validated through neurotransmitter-mediated activity, which is deciphered by receptors on the postsynaptic side.

In order to compare the role of two adhesion molecules (NL1 and SynCAM) and two glutamate receptors (NMDAR and AMPAR) on synaptogenesis, heterologous synapse systems were created between neurons and HEK cells expressing various combinations of these proteins (NL1 alone; SynCAM alone; NL1/NMDAR; NL1/AMPAR; SynCAM/NMDAR; SynCAM/AMPAR). These heterologous synapses were then stained for synapsin, and the size of the presynaptic contact (determined by the area of synapsin staining) was compared between the experimental groups. Results show that receptor expression causes the forma-

tion of smaller contacts than when the adhesion molecule is expressed on its own. These results suggest a role for the glutamate receptors in refining synaptic contacts during the process of synaptic validation.

Keywords: Synaptogenesis, Synapse Validation, Neuroligin-1, SynCAM, AMPAR, NMDAR, Heterologous Synapse Systems.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1.0 INTRODUCTION

In order to survive, organisms must be able to receive, process, and transmit information. The field of neuroscience centers on the study of the nervous system, which is the organ system that performs this necessary task. Gaining an understanding of how the Central Nervous System receives information from the periphery and the environment, processes this information to produce thoughts, emotions, and goal-directed behaviors, and transmits commands to the periphery, remains an active area of research. Current studies on the nervous system include molecular and cellular questions examining what genes are expressed in neurons and how they perform their specific task, systems and computational analysis studying how information processing occurs, and cognitive and behavioral neuroscience looking at how nervous system function affects thoughts and behavior.

In the subfield of cellular and molecular neuroscience, an active area of research is the study of synapses, the connections between individual neurons. Since a typical neuron in the Central Nervous System forms tens of thousands of these contacts, it is important to understand how new connections are created. This process is called synaptogenesis. In particular, understanding the role of individual molecular components can help shed light on how the process of synapse formation occurs during normal development, and how this process is altered in developmental diseases like autism and epilepsy that are known to arise due to improper synapse formation. Current techniques to study the role of specific proteins in synaptogenesis typically use knockouts or (if the protein of interest is a receptor

or ion channel) pharmacological inhibition. These experimental approaches have inherent drawbacks. For example, knockouts for key proteins involved in synaptogenesis are often lethal. Furthermore, both techniques have the possibility of producing results that are confounded by compensatory mechanisms, since in the nervous system there are several proteins that have similar (but not redundant) function.

In order to gain an understanding of the role of specific neuronal proteins during synaptogenesis without the risk of genetic compensation, a simplified system is required. Just as our understanding of the complexities of the brain have been drastically furthered through the use of simplified in vitro neuronal culture systems, our understanding of the molecular synaptic dynamics can be furthered through the use of a simplified heterologous system. This system involves expressing only the neuronal proteins of interest (e.g cell adhesion molecules like NL1 and SynCAM, or glutamate receptors like NMDAR and AMPAR) in a non-neuronal cell. When these non-neuronal cells are cocultured with neurons, heterologous synapses form, and can be analyzed. Since non-neuronal cells do not express neuronal proteins, and do not usually form synapses with neurons, they can act as an experimental "blank-slate" that allows for the construction of protein systems of interest using cellular and molecular techniques. This project utilizes this approach to study the specific effects of two cell adhesion molecules, NL1 and SynCAM, and two glutamate receptors, AMPAR and NMDAR, on synaptogenesis

The thesis is organized into seven chapters. Chapter 1 is this Introduction. Chapter 2 will provide Background on neurobiology that is well established, and is relevant for understanding the project. Chapter 3 will provide some of the Theory behind synaptogenesis. Chapter 4 provides the Specific Aims, while Chapters 5 discusses the Materials and Methods used. Chapter 6 provides the Results, and Chapter 7 focuses on a Discussion of these results and also provides Future Directions.

2.0 BACKGROUND

2.1 NEUROBIOLOGY

2.1.1 Neurons and Synapses: Morphology and Physiology

2.1.1.1 Neurons- Cell Morphology and Physiology The majority of information processing and exchange is performed by neurons in the Central Nervous System. Neurons are the specialized cells that are designed to receive and transmit information throughout the body. While these cells vary tremendously in size and shape depending on their specific location and function, they share common characteristics. Neurons generally have three anatomic parts with specialized roles. The soma, or cell body, contains the nucleus and other necessary organelles. Extending off of the the soma are two different types of membranous processes. Dendrites are specialized processes that receive signals from other cells and transmits them to soma. Axons allow the forward propagation of summed information to other neurons in an all-or-nothing signal called the action potential (AP). Axons are typically wrapped in an insulating sheath called myelin, and split at the distal end to form connections called synapses with other neurons. The portion of the axon where the synapse is located is called the terminal bouton. Synapses typically form between axons and dendrites, but axo-somatic and axo-axonal synapses are also possible. An image of a typical neuron is shown in Figure 1 [31, 32].

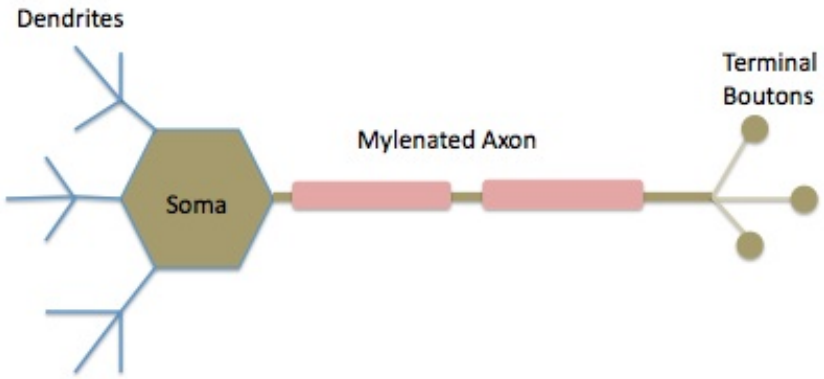


Figure 1: A figure of a typical neuron, with the soma, dendrites, myelinated axon, and terminal boutons labeled. Neurons are the cells that transmit information throughout the body.

Neurons propagate signals within the cell through changes in membrane potential (the voltage difference between the inside and outside of the cell), and transmit signals between cells via a chemical exchange at synapses. The AP is the electrical signal that is used to send information down the axon. These signals serve as the neural "code" through which the nervous system receives and transmits information throughout the body. Action potentials originate at a region called the axon hillock, at the beginning of the axon. They then propagate towards the end of the axon, where the signal reaches the synapse [32, 36].

In addition to the AP signal, neurons also use smaller electrical signals, particular at the dendrites. Neurons maintain a resting potential of around -70 mV by using ion pumps to create and sustain concentration gradients across the membrane, with high extracellular sodium and high intracellular potassium. Additionally, calcium ions, while present at significantly lower quantities than sodium or potassium, have a higher extracellular than

intracellular concentration. Neurons can change their membrane potential through the opening of selectively permeable ion channels. The changes in ion permeability that result from channel opening, coupled with the established concentration gradients, results in ion flow either into or out of the neuron. Sodium ion influx results in a depolarizaiton, or positive change in the membrane potential; potassium ion efflux results in a hyperpolarization, or negative change in the membrane potential. Using this mechanism of ion flow, neurons can create electrical signals. The influx of calcium ions can also trigger biochemical cascades that alter the cell's protein expression and localization, since calcium is an important second messenger molecule [32, 36].

2.1.1.2 Synapses: Neuron-to-Neuron Connections Synapses play a crucial role in synthesizing, integrating, and transmitting information through the nervous system. Information is transmitted in a unidirectional manner from the membrane at the presynapse to the membrane at the postsynapse. The transformation from electrical signal at the axon to a chemical signal occurs via an action potential-dependent release of a chemical called neurotransmitter (NT). The released NT diffuses from the presynapse to ligand-gated ion channels on the postsynapse, opening these channels and causing current to flow through them [32, 36].

Synapses come in two general categories, excitatory and inhibitory. Excitatory synapses cause a depolarization in the membrane potential. Inhibitory synapses cause a hyperpolarization in the membrane potential. The difference is based on the specific NT released by the presynaptic neuron and the receptors that are expressed on the postsynaptic neuron. For example, glutamate is the ubiquitous excitatory NT, while GABA is a common inhibitory NT. This project focuses on glutamatergic, excitatory synapses. Individual neurons have anywhere from one to hundreds of thousands of synaptic inputs, and the postsynaptic neuron integrates these inputs, both excitatory and inhibitory, to decide whether or not to fire an AP depending on whether or not the membrane potential reaches threshold.

In this way, synapses integrate convergent inputs, a crucial function of the nervous system. A figure showing the structure of a synapse is shown in Figure 2 [32, 36].

The specifc proteins involved in synaptic transmission that were examined in this project will be described in more detail in subsequent sections. At the presynapse, these include the proteins synapsin, synaptotagmin, and VAMP; at the postsynapse, these include NL and SynCAM, as well as AMPAR and NMDAR.

2.1.2 Structure of the Postsynapse

The structure of synapses allows for the efficient receipt of chemical signals and transduction of these signals into electrical impulses. The postsynapse is a highly specialized cellular structure that consists of ligand-gated ion channels, scaffolding proteins, and adhesion proteins, which help form protein complexes.This section will provide an overview of the postsynaptic structure; a description of the specific postsynaptic proteins studied in this project will be covered in the following subsections.

Identifying specific presynaptic and postsynaptic constituents, as well as understanding their individual roles in different developmental time points and in normal and diseased states, are critical to comprehending how synapses develop and function. As previously mentioned, synapses that release glutamate are the primary excitatory connections in the human nervous system. At these synapses are two principle families of ligand-gated ion channels: the alpha- amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor (AMPAR) and the N-methyl-D- aspartate receptor (NMDAR). NMDARs are ubiquitously found throughout the brain, while AMPARs are much more variable, and are often missing in some excitatory glutamatergic synapses. Both NMDAR and AMPAR distribution can be varied depending on the activity of the neuron [49].

Structural studies of synapses have shown an electron-dense thickening of the plasma membrane of the postsynaptic cell. This is termed the postsynaptic density (PSD) and contains

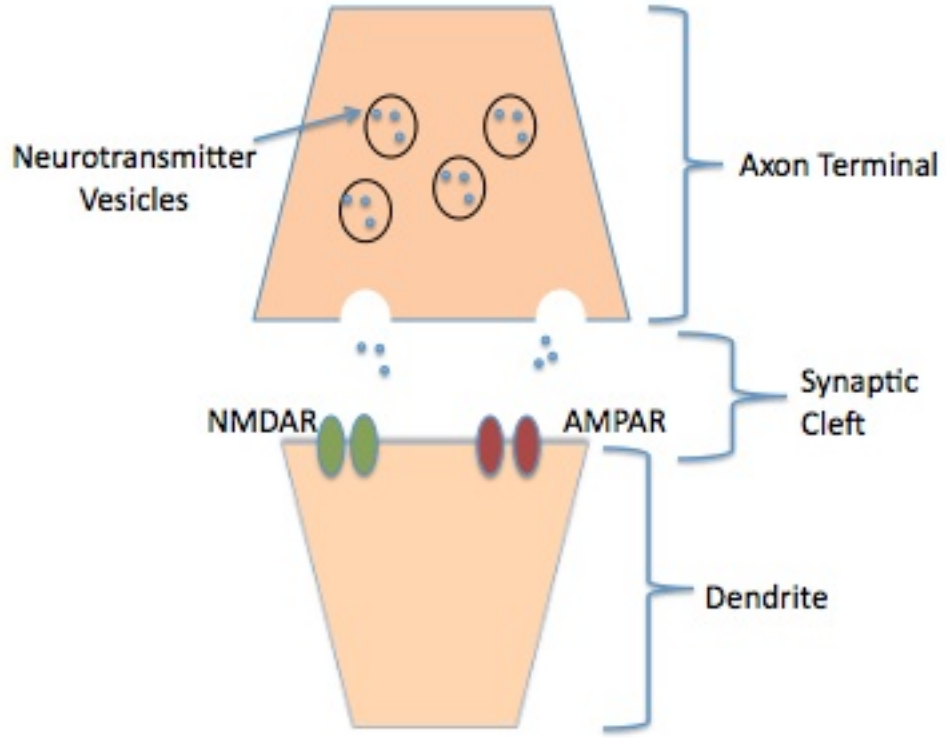


Figure 2: A diagram of a typical synapse, which is the connection between the end of an axon (the presynapse) and the dendrite (the postsynapse). The axon terminal, synaptic cleft, and dendrite are labeled. Neurotransmitter vesicles and receptors are also shown. At a synapse, once an action potential arrives at the presynaptic terminal, the depolarization leads to a calcium influx that triggers the exocytosis of neurotransmitter from the vesicles into the synaptic cleft. The neurotransmitter then bind to receptors on the dendrite, which produce graded potentials at the postsynaptic neuron which allow for the continuation of signal propagation.

the various postsynaptic proteins. Along with receptors, there are scaffolding proteins (e.g. Stargazin and PSD-95) that often contain specific amino acid sequences (called PDZ domains) that bind to other proteins (e.g. AMPAR and NMDAR). This interaction results in the formation of complexes that group the channels, scaffolding proteins, and other key molecules in a tight cluster so that ion flow through the channels can mediate changes at the synapse [45]. For example, through the formation of these complexes, glutamate receptors can transduce presynaptic NT into a signal that affects downstream signal transduction, cytoskeletal anchoring of postsynaptic proteins, and receptor trafficking [49]. The NMDAR forms several of these complexes. One specific example involves the scaffolding protein PSD-95, a protein that appears to play a role in synaptic targeting and clustering of the NMDAR during synaptic validation. The AMPAR also form receptor complexes at the synapse by binding to the PDZ domains of Stargazin, which then binds to PSD-95. Just like for NMDARs, experimental evidence also suggests that these clusters play a role in the synaptic targeting of the receptors [45].

The two sides of the synapse are linked to one another by adhesion proteins. Two examples that are commonly found at glutamatergic synapses include Neuroligin-1 (NL1) and SynCAM [29].

It is important to emphasize when talking about postsynaptic structure that the postsynaptic density is not static, but dynamically regulated on many levels. Recent studies have shown that rapid, short-term changes in postsynaptic molecular composition can occur due to different stimuli, and longer-term changes in the postsynapse are often thought to be implicated in learning and memory. This regulation of the postsynapse can be either long-term potentiation (LTP) or long- term depression (LTD). Current evidence indicates that the AMPAR subunit GluR1 plays a critical role for LTP. This subunit appears to undergo phosphorylation by multiple postsynaptic proteins, including CaMKII and PKC, and this phosphorylation potentiates receptor function [45]. In addition to this mechanism, LTP can occur simply by increasing the number of glutamate receptors at synapses. One

example of this process is the so-called "unsilencing" of "silent synapses." Silent synapses have NMDARs but no AMPARs, and as such are "silent" because the NMDARs have a voltage-dependent magnesium block that prevents ion flow through the channel. Strong experimental evidence has shown that these synapses are "unsilenced" through the recruitment of AMPARs to the synapse- the AMPAR then allows ion flow that depolarizes the membrane, removing the magnesium block [26]. The potentiation of synapses through AMPAR subunit phosphorylation and AMPAR recruitment emphasize how very specific changes to postsynaptic proteins can alter nervous system function.

2.1.2.1 Postsynaptic Adhesion Molecules: Neuroligin-1 and SynCAM Adhesion proteins like NL1 and SynCAM link the pre- and postsynaptic side to one another, providing both structural stability and bidirectional signaling. This project focusses on the role of NL1 and SynCAM in synaptic development. Both proteins play critical roles in neuronal target recognition, as well as synapse formation, stabilization, and maturation during development. These roles will be discussed in detail in the Theory Section. This section will focus on the structure of NL1 and SynCAM, as well as their role in other aspects of synaptic function separate from development. It will also briefly describe the structure and function of neuroligin's presynaptic binding partner, the neurexins.

Neuroligins were identified based on their role as postsynaptic cell-adhesion molecules that are located at excitatory synapses. They bind to the neurexins to form heterodimers at the synaptic cleft. Neuroligins are not capable of homomeric interactions. There are five human genes that code for the neuroligins (NL1-5); each individual neuroligin has a specific neurexin binding partner. NL1 is one of three neuroligins that binds to beta-neurexins. The neuroligin family members show significant sequence homology, but differ more in the intracellular than extracellular domains. This suggests that the extracellular domain is responsible for linking the pre- and postsynaptic sides in a conserved manner, while the intracellular domain is involved in the transport of specific neuroligins to the appropriate

synapses and also in the recruitment of different postsynaptic components to different types of synapses [29]. The structure of NL1 is shown below in Figure 3.

Intracellularly, postsynaptic NL1 links to scaffolding proteins like PSD-95, and is found clustered with NMDAR subunits. NL1 are only present at glutamatergic excitatory synapses and are not found at inhibitory connections like GABAergic synapses [50]. Extracellularly, neuroligins contain a cholinesterase-like domain, the region of the protein where the molecule binds to presynaptic neurexins. Unlike a typical cholinesterase-domain (found in proteins like Acetylcholinesterase), this region on neuroligins does not have enzymatic activity, but is able to mediate the binding of the two adhesion molecules similar to enzyme/substrate interactions [29].

The neurexins themselves are encoded by three genes. These undergo splice variation to produce the two distinct categories of neurexins that are found, the alpha- and beta-neurexins. The two splice variants have what is called an LNS domain, which binds to the cholinesterase- domain of the neuroligins to cause the cell adhesion. While the alpha-neurexin has six LNS domains, the beta-neurexin has only one. In addition, the neurexins contain PDZ binding domains that can potentially recruit presynpatic constituents to the synapse [29].

Functionally, the interaction of NL1 with beta-neurexin recruits the appropriate pre- and postsynaptic molecules (i.e. postsynaptic receptors and presynaptic NT vesicles) to the synapse, and then uses scaffolding molecules to oppose these proteins to one another at the synaptic terminal. All neuroligin members bind to the scaffolding protein PSD-95, which then assembles protein complexes that include both the NMDARs (directly) and AMPARs (indirectly through a second scaffolding protein, Stargazin). The binding of the two proteins may also recruit other regulatory molecules, like kinases, to the synapse, which can then alter synaptic function as needed [29].

SynCAMs, also called IGSF4, are another family of cell adhesion molecules that are studied

in this project. These molecules are part of the immunoglobin superfamily, a large group of cell adhesion molecules. Immunohistochemical studies in drosophila and rats have shown that this is a cell adhesion molecule that forms homomeric interactions. The possibility of heteromeric interactions by SynCAM have not been ruled out, but potential heteromeric binding partners have not yet been identified. The structure of SynCAM is shown in Figure 3. The molecule has three "Ig domains" that bind to other cell adhesion molecules. The intracellular portion of SynCAM contains a PDZ domain that can then bind to other scaffolding and signaling molecules that also contain the domain [55].

SynCAM is not only involved in synapse development and function, but also acts as a tumor suppressor for lung cancer, as an adhesion molecule for sperm cell precursors, and as a cell adhesion molecule for mast cells. Because it was discovered by different researchers independently, the protein has been given three different names in the literature: TSLC1, SgIGSF, and SynCAM [55].

Including its role in synaptic development, which will be discussed in the Theory Section, SynCAM has functions in modulating synaptic transmission. Transfection of SynCAM into hippocampal neurons increases the frequency of spontaneous miniature excitatory postsynaptic potentials, a measure of the frequency of synaptic activity. This increased synaptic activity can be due to either an increase in the number of synapses (a developmental process), or an increase in the frequency of NT release from existing synapses (a modulation process). Immunocytochemical studies measuring the number of synapses, combined with electrophysiological studies measuring synaptic release, show that SynCAM is actually involved in both processes [55].

2.1.2.2 Scaffolding Proteins: PSD-95 and Stargazin As previously mentioned, the scaffolding proteins create complex molecular networks that cluster proteins at appropriate areas in the synaptic terminal. Scaffolding proteins are not studied in this project; however, understanding their role is important in identifying mechanisms of synaptic plas-

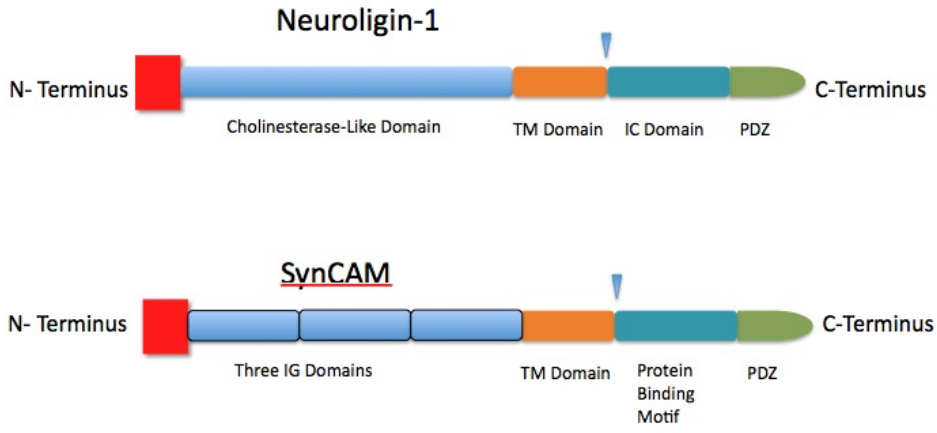


Figure 3: A simplified schematic showing the molecular structure of the two postsynaptic cell adhesion molecules studied in this project: NL1 and SynCAM. The extracellular portion of the protein is shown to the left of the arrow, and the intracellular domain is shown to the right. Both molecules have a transmembrane domain and a PDZ domain. SynCAM has three extracellular IG domains that bind to presynaptic SynCAM, while NL has a cholinesterase-like domain that binds to presynaptic neurexins. SynCAM also has an additional intracellular protein binding domain.

ticity as well as changes in glutamate receptor function. This section will provide a brief overview of two scaffolding proteins often associated with the function of the glutamate receptors, PSD-95 and Stargazin. Since this project also uses a simplified model to avoid potential compensatory mechanisms inherent in biological systems, this section will also cover a few knockout experiments of PSD-95 in mice. The results of these experiments are commonly used as an example of a compensatory mechanisms in neurobiology, since the knockouts produce results that are conflict with in vitro studies and existing LTP models.

The PSD-95 family of proteins is encoded by four genes. The protein contains three different PDZ domains, as well as two other domains important in protein interactions, the SH3 and GK domains. The PDZ domains can cluster together and create multimers that can recruit NMDARs, AMPARs, and a variety of other proteins like potassium channels and kinases, facilitating signal-coupling at the synapse. Glutamate receptor clustering appears to be independent of PSD-95 since mutations to PSD-95 do not affect normal clustering. However, the localization of receptor clusters to the synapse is disrupted by PSD-95 mutations, since in neurons with these mutations many receptor clusters are found extrasynaptically. The interaction by PSD-95 with NL1, which then interacts with beta-neurexin, allows for presynaptic proteins to be located in register with the postsynaptic density. Since the NL-beta-neurexin interaction is critical for neural targeting and synaptic development, it is conceivable that PSD-95 plays a role during synaptogenesis as well [25].

PSD-95 also links to Stargazins, which then bind to AMPARs. Since LTP is characterized by increase in AMPAR currents, one model of LTP is that the calcium influx through NMDARs at the synapse causes downstream signaling that allows for PSD-95 to recruit Stargazins to the synapse, and the Stargazins bring with them AMPAR subunits like GluR1 [25].

While the structural role of PSD-95 and its interactions with other proteins is relatively well

characterized, the functional role of this protein is not well understood. Experiments on PSD-95 are often used as examples of how potential compensatory mechanisms can create results seemingly in conflict with one another. PSD-95 knockout mice have impaired spatial learning and defective LTD, but actually have enhanced LTP [34]. However, an analysis of synaptic currents in in vitro cultures with PSD-95 knockout or overexpression suggest that PSD-95 plays an active role in producing LTP via the recruitment of Stargazin, as previously described. This conflict between the in vitro results and the phenotype of PSD-95 knockouts needs to be reconciled, but it is likely that in the in vivo system, another protein is substituting for the removed PSD-95 and producing an altered response [25].

2.1.3 Overview of the Glutamate Receptors

As previously mentioned, most excitatory activity in the mammalian central nervous system is mediated by the glutamate receptors. These receptors also are involved in the strengthening or weakening of synapses through LTP and LTD. Glutamate receptors are involved in pathophysiology, since excessive activation of these receptors during traumatic brain injury or epileptic seizures can lead to neuronal death by a mechanism called excitotoxicity. Understanding the role of receptor expression and activity during development and disease remains a critical research question [37].

There are two main classes of glutamate receptors- the ionotropic and the metabotropic receptors. Ionotropic receptors, which includes the AMPARs and NMDARs, are a family of ligand-gated ion channels encoded by 18 different genes [33]. The metabotropic receptors are membrane bound receptors that interact with G-proteins to produce second messengers that indirectly produce the postsynaptic response. Because of their mechanism of action, ionotropic receptors mediate "fast" excitatory transmission, while metabotropic receptors result in slower, but more long-term responses due to the cascading effects of second mes-

senger signaling systems [37]. The ionotropic receptors can be divided into three main families on the basis of the specificity of specific agonists: the kainate receptors, AMPARs, and NMDARs (a fourth family, the delta receptors, is sometimes included in the literature). This project focuses on AMPARs and NMDARs.

Each receptor is composed of four subunits. The different types of subunits show tremendous diversity- currently there are four known AMPAR subunits (GluR1, GluR2, GluR3, and GluR4) and seven known NMDAR subunits (NR1, NR2A, NR2B, NR2C, NR2D, NR3A, and NR3B). Each NR2 subunit has its own specific glutamate-binding site, and each NR1 and NR3 subunit has a glycine binding-site. Modeling studies of glutamate receptors suggest that the mechanism by which they open involves the binding of agonist to the subunit, creating a torque on the protein that leads to channel opening [33].

The receptor subunits typically have a large, N-terminus extracellular domain, three hydrophobic transmembrane domains, and an intracellular C-terminus. There is another hydrophobic, region does not make a complete pass through the membrane, but makes a turn within the membrane (called a re-entrant membrane loop) and forms the P, or Pore, domain. The P domain from the four subunits surrounds the water-filled pore that allows ions to pass through the membrane.The presence of this re-entrant loop provides a structural distinction between the glutamate receptors and other receptors found at excitatory synapses, such as the GABA and acetylcholine receptors [11].

Structural schematics for both the NMDAR and AMPAR are shown in Figure 4.

2.1.3.1 Postsynaptic Receptors: AMPA Receptor Among the three families of glutamate receptors, AMPARs are the most critical in mediating the fast responses at excitatory synapses in the mammalian CNS. This project studies one class of AMPARs, the homomeric GluR1 AMPAR. This section will cover the molecular structure, biophysical properties, pharmacology, and physiology of these receptors.

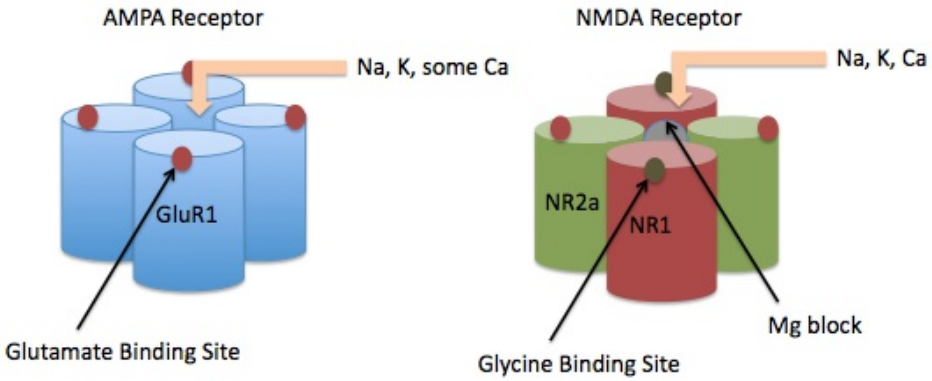


Figure 4: A simplified schematic showing the molecular structure of the two glutamate receptors studied in this project, the AMPAR and NMDAR. These receptors bind to the neurotransmitter glutamate and then open to allow the flow of specific cations through the channel. The separate subunits, as well as the neurotransmitter binding sites and ion permeabilities, are labeled. Also shown is the magnesium block, which prevents the NMDAR from being activated unless both neurotransmitter and a postsynaptic depolarization are detected.

As previously mentioned, the AMPARs is composed of four separate subunits, which are all approximately 900 amino acids in size and have about 70 % sequence homology between them [37]. AMPARs can be either homomeric (composed of identical subunits) or heteromeric (composed of different subunits). Immunoprecipitaiton studies have found that AMPARs are typically GluR2/GluR1 or GluR2/GluR3 heteromers, but there are GluR1 homomers present in various different brain regions. The distribution of different types of AMPARs vary not only between different brain regions, but also within individual neurons themselves; studies combining immunohistochemical staining for specific AMPAR subunits and electrophysiological measurements of currents have shown AMPAR with different subunits combinations within the same neuron [11].

The subunit compositions of a specific receptor results in profound changes in the biophysical properties and physiological function of the receptor. AMPARs are distributed broadly through the CNS, although there are often differences in the amounts in different brain regions. High levels of AMPARs are found in the hippocampus, cerebral cortex, deep cortex, brainstem, and basal ganglia. Lower levels were found in other regions like the cerebellum. In situ hybridization to determine the subunit composition of AMPARs at different brain regions show that in the hippocampus the receptors are composed of GluR1, GluR2, and GluR3 (including GluR1 homomers, used in these experiments), while in cerebral cortex all four subunits are present, with GluR2 found in all cortical layers and GluR1, GluR3, and GluR4 expression differing between layers [37].

AMPARs are permeable to sodium and potassium ions. Studies expressing combinations of receptor subunits in HEK293 cells and measuring ionic currents show that homomeric GluR2 receptors have almost no calcium ion permeability, while homomeric GluR1, GluR3, or GluR4 channels are highly permeable to calcium ions and allow a substantial inward flux of the ion. For example, the ratio of permeability to calcium compared to permeability to sodium and potassium in homomeric GluR1 is 2.34 [11]. Heteromeric channels with GluR2 combined with either GluR1, GluR3, or GluR4 results in channels with little calcium

permeability [23]. These experiments suggest that it is the GluR2 subunit that prevents calcium flow through this channel. The amino acid within the P loop of AMPARs that determines calcium permeability is a charged arginine in GluR2 subunits but a noncharged glutamine in the other subunits. Interestingly, this arginine is NOT encoded in the GluR2 DNA, but introduced into the protein by RNA editing. In mature adult brains, all GluR2 is expressed in this edited form but in embryonic brains, a percentage of GluR2 subunits do not undergo RNA editing and therefore form AMPAR that has calcium permeability [37].

AMPARs have short time constants for both opening and closing, reflecting its role in mediating fast synaptic transmission. The receptor closes more rapidly than the time it takes glutamate to diffuse out of the synapse, and so the mechanism of closing is likely due to desensitization (closing of the receptor even in the presence of agonist) rather than deactivation (removal of the agonist) [11].

Pharmacologically, there are several molecules that act as antagonists to the AMPAR; these include CNQX, DNQX and YM90K. These molecules are often used by researchers to block AMPAR currents [37].

Two final points to note about the AMPAR involve the functional significance of calcium permeability through some of these receptors, as well as the pathophysiology of AMPAR. Some cells in the CNS, particularly hippocampal interneurons and neocortical interneurons, have AMPAR with high calcium permeability. Experimental analysis of the receptor types in these cells using RT-PCR has shown that these cells contain a decreased amount of the GluR2 subunit relative to the other AMPAR subunits. This is expected since GluR2 confers calcium impermeability. Researchers hypothesize that, in addition to the NMDARs, these calcium- permeable AMPARs (i.e. homomeric GluR1 receptors) play a role in providing calcium influx triggered by synaptic activity; calcium can then function as a signaling molecule and play a role in modulating synaptic function. In line with this hypothesis, hippocampal neurons containing calcium-permeable AMPARs had markedly

enhanced LTP, a process believed to involve calcium influx into the cell. Furthermore, this LTP could be induced in the presence of blockers for NMDARs, which is the receptor typically thought to be involved in synaptic plasticity. These results suggest that the calcium influx through AMPARs play a critical physiological role, particularly in learning and memory. There is also overwhelming evidence that excessive calcium influx can occur through calcium-permeable AMPARs during brain injury, and AMPAR antagonists, like CNQX and DNQX can prevent neuronal death caused by ischemia in the hippocampus [37].

2.1.3.2 Postsynaptic Receptors: NMDA Receptor The NMDAR produces postsynaptic responses that are slower and more prolonged than the AMPAR, and because of their molecular and biophysical characteristics are often thought to be implicated in processes like learning and memory. This project uses the NR1/NR2A type of NMDAR. This section will provide a brief overview of the molecular structure, biophysical properties, pharmacology, and physiology of these receptors.

The fundamental differences between the AMPAR and NMDAR that result in their different physiological roles include the NMDARs increased permeability to calcium ions, slow kinetics, and voltage-dependent magnesium block. As previously mentioned, there are seven genes for NMDAR subunits (NR1, NR2A, NR2B, NR2C, NR2D, NR3A, and NR3B). Initial experiments on Xenopus oocytes found that transfection of just the NR1 subunit was sufficient to produce functional receptors. However, this same experiment in mammalian cells found that NR1 homomers only produce a small postsynaptic response, and NR2 homomers do not form functional channels. Eventually, it was found that an endogenous Xenopus protein associated with NR1 to form functional channels, and it is now accepted that in mammalian cells, NMDARs cannot form homomeric channels. It is hypothesized that two NR1 subunits must be coupled with two NR2 subunits (though it is not necessary to have the same two NR2 subunits in a given channel) [14]. Because

all NMDARs express the NR1 subunit but differ in their NR2 composition, the NR1 can be thought of as a "fundamental" subunit while the NR2 serves a more modulatory role. From a genetics perspective, the sequence homology between the NR1 and NR2 subunits are quite low compared to the homology between different NR2 subunits (18 % compared to 50 %) [11, 37].

Immunohistochemical stainings for the NMDARs have shown that these receptors are distributed throughout the mammalian central nervous system, with a particularly large amount found in the forebrain and hippocampus. In situ hybridization shows that NR1 mRNA is found throughout the central nervous system, while the four NR2 subunits have much more specific distributions. The NR2A is found throughout the brain, with a particular emphasis on the cerebral cortex, hippocampus, and cerebellum. The other three subunits are found in the forebrain, cortex, hippocampus, basal ganglia, thalamus, brain stem, and cerebellum. The expression of NMDAR subunits changes during the course of development; in situ hybridization studies have shown that the NR2B and NR2D receptors are primarily expressed before birth, while the NR2A and NR2C show increased expression after birth. This shift is most substantial in the cerebellum, and is dependent on activity. In neurons that are cultured in vitro, the switch from NR2B to NR2A is normally seen, but NR2B expression remains high if the culture is chronically exposed to TTX, a sodium channel blocker that prevents action potential generation. It has been hypothesized that this shift helps tailor the receptor that is expressed in different brain regions to that region's neurological task. [37].

Like the AMPAR, the NMDAR is permeable to monovalent cations like sodium and potassium. However, the NMDAR differs from the AMPAR in its permeability to divalent cations. The receptor shows substantial permeable to calcium, and is even more permeable than homomeric GluR1 AMPARs. While the ratio of permeability of calcium to sodium and potassium in the GluR1 R is 2.34, in the NMDAR it ranges from 3.10-11 [11]. Experiments expressing NMDARs in HEK cells, bathing the cells in media containing

physiological amounts of cations, and measuring the percentage of ionic currents through the channel show that about 10 % of the total NMDAR current is carried by calcium. This relatively large calcium influx through the channel functions as a second messenger molecule at postsynapses, altering synaptic structure and protein function in a long-lasting way [37, 14].

NMDARs also show a voltage-dependent magnesium block- unless the membrane potential is slightly depolarized, the receptor pore is blocked by magnesium ions.The mechanism of this blockage can be explained by understanding the structure of the receptor. At the resting voltage the magnesium ion in essence gets "stuck" inside the channel, blocking current flow through the receptor. Because magnesium is a positive ion, a hyperpolarized membrane would attract the ion, and so it would be more likely to move towards the inside of the channel; depolarizing the membrane, which would make the inside more positive, would result in less of an attractive force of the magnesium ion to the inside of the cell, and this would result in the movement of the ion out of the pore. The voltage block is much stronger for NR1/NR2A and NR1/NR2B channel than the NR1/NR2C and NR1/NR2D channels; in NR1/NR2A or NR1/NR2B channels, the block is removed only at -25 mV, compared to -45mV for the other two types of channels [37].

In order for the NMDAR to be activated, the receptor needs to bind to both glutamate and glycine. In physiological systems, it appears that glutamate is the neurotransmitter released by the presynapse, while sufficient glycine is present in the extracellular solution for partial occupation of the glycine-binding site. Research has shown that mutations on the NR1 subunit reduced the receptor's affinity for glycine, while mutations on the NR2 subunit reduced the affinity affinity for glutamate. Thus, the NR1 binding site is for glycine, while the NR2 is for glutamate [11, 37].

In experiments, pharmacological agents used to block NMDAR include MK-801, AP5, CNQX, nitric oxide, and D-APV [37].

The kinetics of the NMDAR are characterized by both slower opening and closing times than the AMPAR. However, glutamate binds to the NMDAR with much greater affinity than it binds to the AMPAR [11].

Three critical characteristics are crucial for the role of the NMDAR in synaptic plasticity: the Mg block at resting potentials, the significant permeability to calcium, and its slow receptor kinetics. This allows the receptor to function as a molecular "coincidence detector" that only opens in response to glutamate application following an initial depolarization, and produces calcium ion influx ; calcium then acts as a second messenger molecule in various biological pathways. AMPARs and NMDARs have been found to be colocalized and are often simultaneously activated by glutamate released by the presynapse. One model of synaptic plasticity then involves the AMPAR, with its lower affinity to glutamate but faster kinetics, producing a "quick" response to presynaptic activity, while the NMDAR, with its stronger affinity for the neurotransmitter but slower kinetics, producing a more prolonged responses that can ultimately alter synaptic strength [37]. The calcium influx could play a role in these changes at the synapse, since both the NMDAR and AMPAR are phosphorylated by CAMKII, a kinase that is activated by calcium. This phosphorylation results in increased current through the AMPAR, which may account for the increased current flow at the postsynapse following LTP [11].

Finally, NMDARs are critical in both development (discussed in the Theory Section) and injury. Traumatic brain injuries, strokes, and seizures often result in the overactivation of the NMDAR, which causes excessive calcium influx that ultimately leads to neuronal death. There is also some evidence to suggest that NMDAR activity is involved in neurodegenerative disorders [11].

2.1.4 Structure and Function of the Presynapse

This section will provide a brief overview of the structure and function of the presynapse as it relates to synaptic transmission. For the purposes of this project, the key presynaptic proteins that are immunolabeled are the priming protein synaptobrevin/VAMP, the calcium detection protein synaptotagmin, and the synapsins, which are involved in maintaining a reserve pool of NT vesicles.

The region on the presynapse that is analogous to the postsynaptic density is called the presynaptic active zone. The active zone contains the voltage-gated calcium channels that are opened by the incoming action potential, as well as the NT vesicles. These are attached to specific proteins to form the SNARE apparatus, and fuse with with the plasma membrane and release NT into the synaptic cleft by exocytosis following calcium influx. In addition to the active zone, there is a region of the presynapse called the reserve pool, where excess NT vesicles are stored and recruited to the active zone if repeated neuronal activity depletes the NT vesicles there. The molecules that are involved in forming the reserve pool are the synapsins. Experimental disruption of the synapsin genes have been shown to reduce the number of NT vesicles in the reserve pool [20]. At the active zone, the proteins that are involved in the docking and release of NT vesicles are collective called the SNARE proteins, and include VAMP. An additional protein called synaptotagmin is involved in detecting the increased intracellular calcium and initiating vesicle release.

Because the active zone is involved in vesicle exocytosis and re-uptake, an extensive cytoskeletal network is also present, with a variety of cytoskeletal-associated proteins present at the presynapse. These proteins, which include Munc, Rim, Piccolo, and Bassoon, are involved in a variety of functions, including vesicle docking, vesicle fusion, NT reuptake, and the modulation of the reserve pool [20].

Just like the postsynapse, the presynapse is a dynamic structure. Specifically, changes in NT release occur during LTP, and these are mediated by the synapsins. Synapsins bind to

a variety of presynaptic proteins, and play a role in the phosphorylation dependent increase in NT release following AP generation. Synapsins also play a role in maintaining, and often increasing the size of, the "reserve pool" of NT vesicles for use during frequent presynaptic activity. Changes in the number of vesicles in the reserve pool correspond to changes in the stability and the strength of the synapse, and so labeling synapsin at synaptic terminals can provide insight into changes in presynaptic morphology and function.

A schematic diagram of the presynapse can be seen in Figure 5.

2.2 MOLECULAR BIOLOGY

This project utilizes transient transfection to introduce neuronal genes encoded on plasmids into non-neuronal cells. The non-neuronal cells then express the proteins coded for by the DNA sequence. Protein expression can then be confirmed using Western Blots and Immunocytochemistry.

This section will review the basic molecular biology used in the experimental methods of this project, including plasmid identification, plasmid purification, mammalian cell transfection, Western Blotting, and immunocytochemistry.

2.2.1 Plasmids and Transfection

Plasmids are circular strands of DNA typically found in bacteria (and also some yeast) that are separate from the chromosomal DNA. Plasmid size can be as large as 1000 kilobase pairs, and in nature they provide a mechanism for gene transfer within an existing bacterial colony without the need for reproduction. In nature, plasmids either encode for genes that provide resistance to antibiotics or other toxins or provide the means to fill an environmental

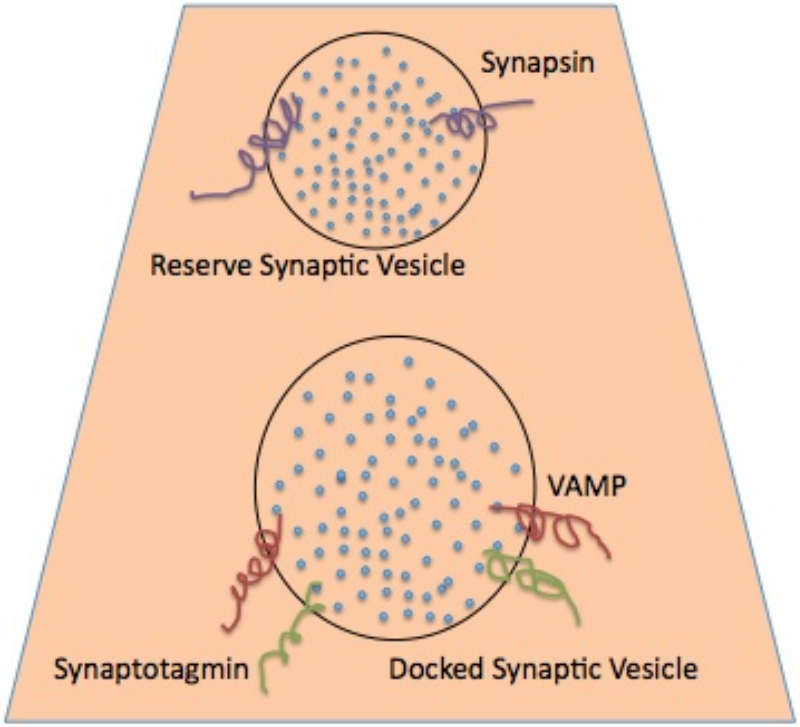


Figure 5: A diagram of the presynaptic active zone. Synaptotagmins and VAMPs are shown, while synapsins are not because they are not located in the active zone, but rather regulate the reserve pool.

niche (like the ability to fix elemental nitrogen). Molecular biology research often involves introducing proteins of interest into cells that normally do not express these proteins, or express them at low levels. This process involves incorporating genes of interest into existing plasmid DNA, growing the plasmids in bacteria, harvesting the plasmids, and then introducing them into the desired cells using techniques called transfection [28].

The structure of a typical research plasmid contains several specific sites within the DNA sequence, including: an origin of replication, where bacteria could begin to replicate the DNA; mammalian promoters, which allow the DNA sequence following the region to be transcribed into RNA using the transfected cell's DNA machinery; several restriction sites, which are specific nucleotide sequences that are cut by restriction enzymes and can be used for both gene insertion and plasmid identification; multiple cloning sites, which are short regions containing several restriction sites that allow the desired DNA sequence to be introduced into the plasmid; and a gene encoding for antibiotic resistance, which allows for the identification of bacteria containing the plasmid, making purification easier [28]. A structure of a typical plasmid is shown below in Figure 6

Plasmids for different genes can be prepared by digesting the multiple cloning sites with appropriate restriction enzymes to create a gap in the plasmid, then inserting the gene of interest into this cloning site using a ligase enzyme. Restriction enzymes recognize and cut specific DNA sequences. For example, EcoR1 recognizes GAATTC and cuts between the G and the A. This creates "sticky ends," where the nucleotide sequence has no complementary base pair attached, and this can be used to insert a gene sequence that has matching "sticky ends." As an example, digesting a plasmid with EcoR1 would expose a cloning site with G and AATTC sticky ends; these can then be used to insert a gene with the sticky ends C and TTAAG [28]. In this project, the plasmids were simply obtained from other laboratories that designed them beforehand.

Once plasmids are either created or obtained, they can be introduced into bacteria (usually E. Coli) so the cell machinery can duplicate the DNA, amplifying the number of copies of

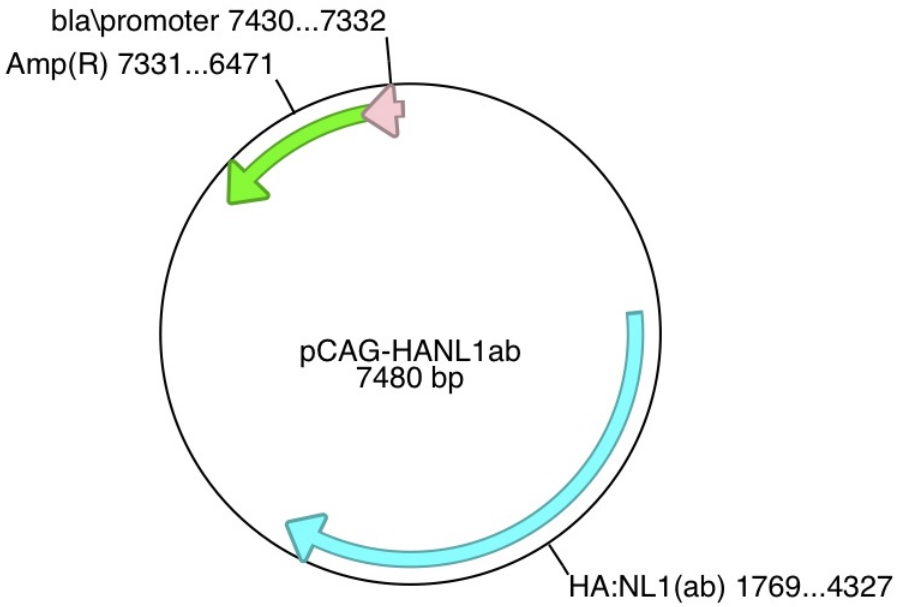


Figure 6: A diagram of the plasmid for NL1 that was used in this experiment. The promotor, gene for antibiotic resistance, and gene coding for NL1 are shown.

the plasmid. This process is called "transformation," and was done in this project using the heat shock method. In this process, the plasmid and bacteria are incubated together at a low temperature in an ice bath. Then, the mixture is placed in warm water, just above physiological temperature (42C). This sudden increase in temperature causes pores in the bacteria to suddenly increase in size, allowing the DNA to enter the bacteria [28].

The bacteria is then allowed to grow on medium supplemented with the antibiotic that the plasmid encodes resistance for; only bacteria that have actually incorporated the plasmid will be able to survive and create colonies. Individual colonies can then be selected and cultured, and the DNA and protein within the cell can be obtained by alkaline lysis. This is a process where a strong base (e.g. sodium hydroxide) breaks the cell wall. A detergent (e.g. sodium dodecyl sulfate) then dissolves the plasma membrane. The DNA can then be separated from bacterial proteins and chromosomal DNA using column purification, which separates components based on charge and size. The DNA can then be further purified using ethanol precipitation or phenol/chloroform extraction, which separates the components based on solubility [28].

The identity of this amplified, purified plasmid can then be determined use restriction digests and gel electrophoresis. This process involves using restriction enzymes to cut the plasmid at specific sites, resulting in DNA fragments. The cut DNA can then be loaded into an agarose gel. Agarose gels are composed of crosslinked polymers with small pores for the DNA to pass through. When a voltage is then applied to the gel, negatively charged DNA fragments will travel through the gel at different speeds, with the larger, more negative fragments moving more slowly through the gel. The number and size of the fragments can then be compared to what is expected in order to confirm the plasmid identity. Once the plasmid identity is confirmed, plasmid purification can occur on a much larger scale to obtain several hundred milligrams of DNA [28].

The obtained DNA can then be introduced to mammalian cells using a technique called transfection. In this experiment, the mammalian cell used is the HEK293 cell, which will

be discussed in more depth in the Theory Section. A variety of different techniques can be used to introduce DNA into these cells, including using polycations, calcium phosphate, liposome fusion, microinjection, electroporation, and protoplast fusion. However, all of these techniques either are toxic to the cell, or have a poor efficiency. Because of these issues, lipofectamine was used as a transfection agent. This is a cationic lipid that has been shown to form lipid-DNA complexes that completely entrap the DNA and then fuse with the cell membrane, producing transfections that are 100 times more efficient than other techniques. There are problems with cell toxicity, but these can be avoided by removing the lipofectamine agent from the media after the transfection is complete [15].

2.2.2 Western Blotting and Immunocytochemistry

Once HEK293 cells were transfected with the selected DNA, both the expression and the appropriate localization of the protein had to be confirmed. The two methods that were used to do this were Western Blotting and immunocytochemistry. This section will discuss these two experimental methods.

2.2.2.1 Western Blotting Once the transfection has occurred, the cell population can be collected using mechanical scraping, placed in a buffering solution, and then lysed using detergent to break apart the cell membrane. The contents are then centrifuged to separate the protein from the remaining cellular components. During the cell lysis stage, protease inhibitor coctakils, which contain a variety of proteolysis inhibitors, are used to prevent unwanted degradation of the protein sample [2].

The concentration of proteins within the sample can be determined using UV absorption. Amino acids containing aromatic rings absorb UV light at 280 nm, and so the absorbance at this wavelength can be used to calculate the protein concentration. Once this is done, a standard amount of protein (typically 1-3 ug/uL, but ranging from 1-50 ug/ul) is loaded

into a polyacrylamide gel, which is a gel composed of crosslinked polymers that are larger than agarose, allowing proteins to pass through. These gels also contains a detergent (e.g. SDS). The detergent is needed because, unlike DNA, proteins have irregular structures and in order to separate them out strictly based on chain length, the polypeptide chain must be denatured. Using SDS also normalizes the charge of the protein to its mass; since SDS is negatively charged, and coats the protein, the charge of the SDS-protein complex is no longer based on the protein's amino acid sequence but instead proportional to the size of the polypeptide [5].

Once the proteins are loaded into the gel, a voltage is applied across the gel and, based on the same principles that agarose gels separate DNA, the protein moves through the polyacrylamide gel, separating based on size so that the smaller proteins move more quickly through the gel while the larger ones move more slowly. Once the proteins have been separated onto the gel, the proteins are transferred onto a PVDF membrane. This transfer is done because the membrane is easier to handle than the gel. The transfer occurs by sandwiching the membrane to the gel vertically and then running a current in the direction from the gel to the membrane. The current pulls the proteins from the gel into the membrane, maintaining the organization that the proteins had on the actual gel [52].

Once the proteins are transferred to the membrane, a solution must be added to block nonspecific binding sites. This is because the membrane readily binds proteins, and so adding antibodies (which themselves are proteins) to probe for the polypeptide of interest would ultimately lead to false positives on the membrane. Typically, a buffered solution of either 5 % bovine serum albumen or 5 % powdered milk is used to coat the parts of the membrane that do not contain sample protein. After this step, a buffered solution containing antibodies against the protein of interest are added at a concentration of 0.5 to 5 ug/mL and the membrane is incubated for 1 to 3 hours. The primary antibody is produced by a host animal (e.g. rabbit, goat, or chicken) and directed against a specific part of the protein of interest, called an epitope. The epitope can either be an inherent

part of the protein of interest, or can be a small peptide fragment (called a "tag") that is artificially added to the gene before incorporating the gene into the plasmid. This allows for the probing of transfected protein separate from endogenous expression [5].

A secondary antibody is then added. This antibody is directed against epitopes specific to the primary antibody's host animal, and also contains an enzyme like horseradish peroxidase, which produces light when a chemiluminescent substrate is added. After another incubation, the light produced by the interaction of the chemiluminescent substrate and the horseradish peroxidase can be recorded onto film paper in order to determine the potential presence of the protein of interest [5].

2.2.2.2 Immunocytochemistry Another method to determine the expression of a protein in a cell is immunocytochemistry. The added benefit of using this technique is that the actual localization of the protein within the cell can be determined. For this project, this technique can be used to not only confirm that membrane proteins are being expressed, but also that they are being targeted to the plasma membrane. This is essential since the heterologous synapses are formed on the membrane of the non-neuronal cell, and so the proteins of interest need to be targeted to the cell surface.

Immunocytochemistry is similar to standard immunohistochemistry protocols except the staining is done on dissociated cells rather than sliced tissue. Typically, the cells are seeded onto a coverslip either before or after transfection so that the slip can be placed on a microscope slide after staining for easy viewing.

For this procedure, the proteins are first fixed, which allows the cells to be as close to the biological structure as possible by preventing enzymatic destruction, contamination, and structural degradation of the sample. Fixation is achieved either with ice-cold methanol or 4 % paraformaldehyde. In this project, paraformaldahyde is used as the fixative; this requires the sample to be permeabilized as well, so that the cells in the sample have

holes within the plasma membrane for antibodies to reach intracellular proteins. This is achieved using a gentle detergent like Triton-X100. After permeabilization, the cell sample undergoes a process similar to Western Blotting. The sample is exposed to 5 % bovine serum albumin to block non-specific binding sites, then exposed to primary and secondary antibodies for the protein of interest. Just like in Western Blots, the primary antibodies are produced by a host animal and directed against an epitope of the protein of interest (again, either a peptide segment intrinsic to the protein, or a tag that is artificially inserted). The secondary antibody is directed against specific epitopes for the host animal of the primary antibody. In the case of immunocytochemistry, the secondary antibodies do not contain horseradish peroxidase, but fluorescent tags instead. These tags are small polypeptide sequences that absorb light at a specific wavelength, and emit light at another. For example, some antibodies emit green light at about 488 nm, while others emit red light at about 555nm. In this way, immunocytochemistry can stain and differentiate two proteins of interest simultaneously. The samples can then be viewed under a microscope to determine protein expression and localization [42].

2.3 MICROSCOPY

Once cells are stained by immunocytochemistry, microscopy is used to examine the samples for fluorescence. In this project, two forms of microscopy were used- standard bright field microscopy and confocal laser scanning microscopy. This section will provide a brief overview of these two techniques.

In bright field microscopy, the sample is illuminated using a light from below the slide and observed through an objective above the slide. Fluorescent probes can be detected by shining the excitation light from below the sample using a filter that absorbs all wavelengths except the wavelength of excitation, and observing the emitted light from above. This

setup can reach magnifications of up to 1000x, but in standard laboratory settings typical magnifications are in the 40-63X range, and are used as an initial observation because of its simplicity. It has low contrast and resolution, and so it cannot be used for more advanced fluorescent analysis [38].

Confocal laser scanning microscopy is a more advanced viewing system that allows for increased resolution and contrast. In this technique, the light source is a laser, and a pinhole eliminates light that is out-of-focus from the plane that is being viewed. By taking images of several planes through the sample, a 3-dimensional image can be reconstructed by superimposing the slices on top of one another, pixel-by-pixel, using a computer. By adjusting the frequency of light emitted by the laser, the microscope can produce light with the wavelength of excitation for fluorescent proteins [38].

3.0 THEORY

3.1 EARLY DEVELOPMENTAL NEUROBIOLOGY- SYNAPTOGENESIS

The structure of the pre- and postsynapse were discussed in previous sections. This project centers on synaptogenesis, which is the formation of synapses. Each neuron has anywhere from one to several hundred thousand connections. How a neuron forms and maintains these synapses is one main focus of neurobiological research. The formation of synapses typically involves two general phases: the structural binding of the two neurons and the recruitment of necessary synaptic components (a process termed synapse formation or initiation); and an activity-dependent stabilization or elimination (termed synapse validation). Synapse validation not only eliminates unneeded synapses, but also creates appropriate circuits and topographic maps. Both of these phases require proper communication between the axon and dendrite [**?**]. This section will provide a detailed overview of the process of synaptogenesis, with separate subsections for synaptic initiation and synaptic validation. The specific roles of NL1 and SynCAM, as well as NMDAR and AMPAR, will also be discussed in subsequent sections.

3.1.1 Synaptic Initiation

This section will provide an overview of the process of synaptic initiation, from the contact of axons and dendrites through the recruitment of pre- and postsynaptic proteins.

Synaptogenesis in the CNS begins when the axon of one neuron elongates outward and initiates contact with a potential target. Real-time imaging studies have shown that this elongation is an extremely dynamic process, with both the axon and dendrite displaying substantial motility, migrating outward and then retracting [9]. In order to create a synapse, the two cells must create an initial contact that has stable sites of adhesion. This stable structure then allows for the beginning of synapse formation and the development of pre- and postsynaptic specialization. While other simpler synaptic structures like the neuromuscular junction have clearly separate processes for target identification and synapse induction, with specific molecules that have unique roles during each phase, the demarcation between the identification of potential synaptic targets and the induction of synapse formation is often unclear at CNS synapses. For this reason, it is often believed that these processes are complementary, and that both are mediated by a variety of cell adhesion molecules. Among these are the cadherins and the neuroligins [19].

The cadherins are cell adhesion molecules that are found throughout the body; the specific variant of cadherins found at synapses are termed N-Cadherins. These proteins are located at sites of both spontaneous and activity-dependent synaptogenesis. However, these proteins do not appear to be required for synapse formation, since the elimination of N-cadherins using siRNA does not alter either excitatory or inhibitory synaptogenesis. The interesting result from studying N-cadherin knockouts is that the presynaptic binding partner for these proteins, the beta-catenins, are not lost at synapses, suggesting that there are other cell adhesion molecules that are filling in as a compensatory mechanism in knockout mice [19].

The neuroligins also play a critical role in this developmental process. The fact that neuroli-

gin expressed in non-neuronal cells has been shown to initiate the formation of presynapses on contacting axons, and the discovery that this synapse formation is eliminated by the addition of soluble beta-neurexin, supports the belief that the neuroligin-neurexin interaction is critical for the initial formation of synaptic connections. However, there is a definite lack of clarity regarding the postsynaptic molecular mechanisms involved in this process. For example, the scaffolding protein that binds to neuroligin, PSD-95, is still targeted (along with glutamate receptors) to the synapse during the initiation phase even if the domain on PSD-95 that binds to neuroligin is mutated [19]. Furthermore, the mechanism of neuroligin targeting to the synapse is not well understood; the initial hypothesis that PSD-95 brings these proteins to the synapse has been disproved by research that reveals that neuroligin targeting does require the extracellular domain of the protein but does not require the domain that binds to PSD-95 [13].

In a previous section, the variety of genes encoding for neuroligins and their binding partner, the neurexins, was mentioned. The importance of several different types of cell adhesion molecules is believed to be critical during synapse initiation, because the complementary expression of cell adhesion molecules by two neurons can allow for the formation of very specific contact. Since the mechanism behind how specific sets of synapses form is not known, a "lock-and-key" hypothesis is an attractive model to explain the variety of different synapses that are formed in the CNS [19].

Once the contact between the two neurons has formed, both cells recruit the appropriate molecular constituents to the synaptic junction. The recruitment of postsynaptic glutamate receptors is mediated by three different proteins: NARP, the EphB receptors, and Syndecan. NARP has been shown to promote AMPAR clustering after it is released from the postsynapse into the extracellular space. It is hypothesized that this protein binds to the AMPAR on the extracellular domain and forms multimers that cause the receptor clustering. It is not known what the mechanism of this clustering is, or what effect this clustering has on the developmental process. The EphB receptors bind to the presynaptic

adhesion molecule called the ephrins, and induce the clustering of the NMDAR that is initiated by the binding between the extracellular domain of the NR1 subunit and the extracellular domain of the EphB receptor. While the Ephrin B proteins are not implicated in synapse formation, they are aggregated shortly before the clustering of the NMDAR. This suggests that they play a role in "coupling" the clustering of pre- and postsynaptic proteins so that they occur in sync during synaptogensis. Finally, syndecans are proteoglycans that are shown to enhance the maturation of the postsynapse [19].

Following the induction of synapse formation, the presynaptic release machinery is rapidly assembled, and within two hours of contact, presynaptic proteins like VAMP, synaptotagmin, and synapsin are located at the synaptic terminal [9]. Experiments have shown that the ability to release neurotransmitter is preceded by the recruitment of these proteins to the presynaptic active zone, and detailed studies suggest that all of the neurotransmitter vesicles, as well as the necessary proteins for exocytosis, are transported on two precursor vesicles to the nerve terminal. These vesicles also contain the scaffolding proteins that link the presynaptic active zone proteins together [19].

Unlike the presynapse, the postsynaptic receptor complexes are synthesized de novo, with each protein in the complex recruited to the synapse independent of one another. This can occur either simultaneously with presynaptic differentiation (e.g. in some areas of cortex) or following presynaptic differentiation (e.g. in some areas of the hippocampus). Imaging studies have shown that the scaffolding proteins like PSD-95 are actually recruited in a gradual manner before the glutamate receptors. The exact role of individual scaffolding molecules is unclear, and is complicated by the inherent complexity of the neurobiological system. For example, PSD-95 knockouts do not have any alterations in postsynaptic assembly during synaptogenesis, likely because other molecules in the postsynaptic density are filling compensatory roles [**?**]. However, these neurons do have altered functional synapses (as previously discussed) so the various scaffolding proteins are not playing redundant roles.

One final point to note about synaptic initiation is that imaging and electrophysiological experiments have also shown that the AMPAR and NMDAR can be recruited independent of one another, and that the receptor recruitment does not require activity [19]. Imaging studies that examine changes in axon and dendritic morphology as PSD-95 is recruited to the synapse reveals that the changes in protein composition parallel changes in the morphology of neural outgrowth; postsynaptic filopodia change to more elaborate spines, and 80 % of all axon branches occur at sites of stable synapses [9].

A schematic of the process of synaptic induction including both neuroligins and SynCAMs is shown in Figure 7.

3.1.2 Synaptic Validation

This project looks specifically at the effect of glutamate receptor expression on synaptic validation. This section will provide an overview of the general process; subsequent sections will describe both changes in receptor expression that occurs during development and the specific research examining the role of glutamate receptors in synaptic development.

Synapses are actively remodeled following the initial formation of the contact, and many are either stabilized or eliminated in an activity-dependent manner. A schematic diagraming this process is shown in Figure 8.

Several experiments have shown that the elimination of synapses is preceded by a decrease in presynaptic activity, but it is not clear whether this is the only cause of synaptic elimination, or whether activity through different sets of receptors can drive elimination as well. It is also unclear whether specific contacts are formed with the predisposition of being eliminated. Some research has found that a few newly formed synapses do not recruit presynaptic proteins, and these are quickly eliminated. Oftentimes, synapse elimination involves the comparison of activity between different inputs; the postsynaptic neuron stabilizes inputs that have synchronous activity and eliminates synapses that have asynchronous

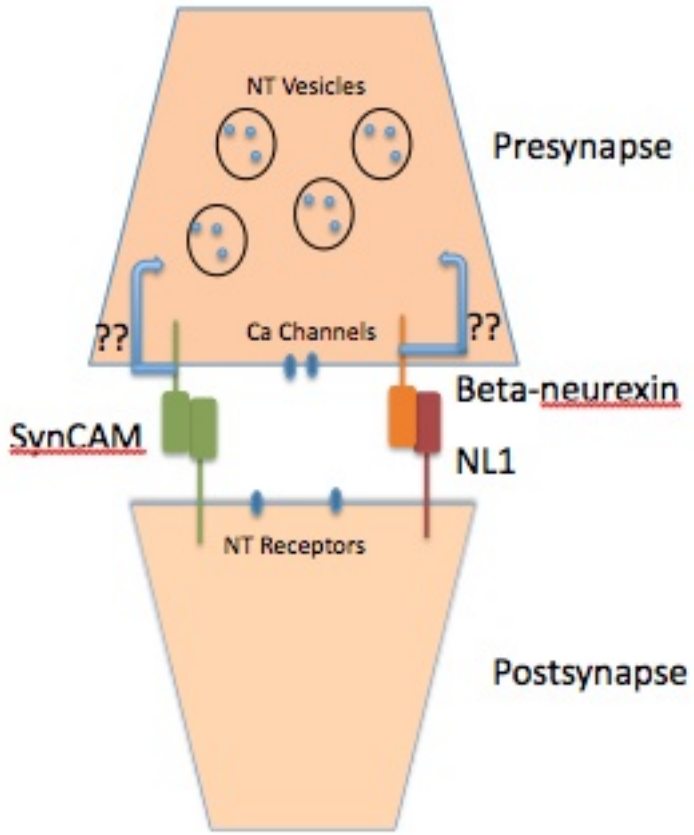


Figure 7: Diagram of synaptic initiation, showing the binding of both SynCAM and NL1 to presynaptic partners, resulting in the recruitment of synaptic vesicles to the presynaptic active zone.

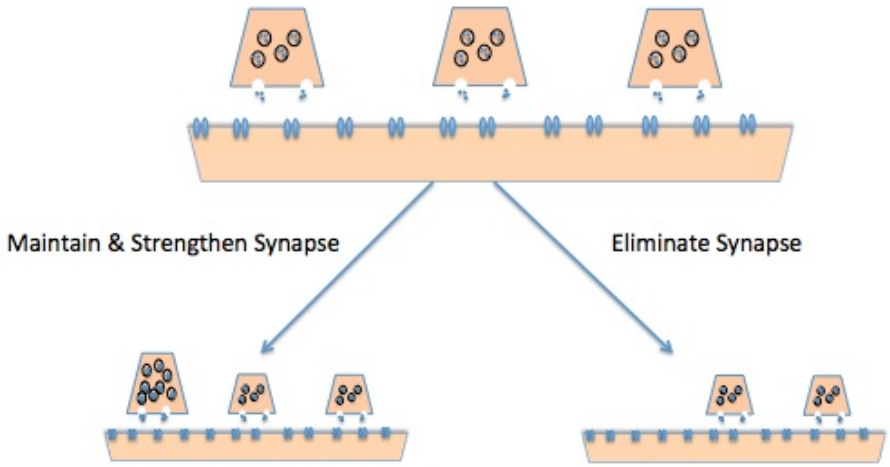


Figure 8: This schematic depicts two types of synaptic dependent validation: synaptic strengthening and synapse elimination. In both cases, presynaptic neurotransmitter is released onto the postsynaptic neuron. Depending on the response by the postsynaptic neuron, there is bidirectional signaling that either strengthens the initial contact (increasing its size and amount of neurotransmitter) or eliminates the contact altogether.

activity. This is summarized by the phrase coined by Hebb, "Neurons that fire together, wire together." Synapse elimination is a rapid process, often occurring within two hours of the initial formation of the contact. Importantly, synapse elimination is a local process that occurs via signaling at the synaptic cleft; the elimination of the contact occurs before the retraction of the axon [9].

It appears possible to have normal synapse initiation in the absence of neurotransmitter release. For example, mice with mutations to proteins required for neurotransmitter release (the SNARE proteins VAMP, syntaxin, or SNAP-25) form synapses that appear structurally normal, and create proper circuitry in various brain regions. In vitro cultures that are chronically exposed to neurotransmitter receptor blockers (CNQX or APV) also form contacts that have appropriate clustering of synaptic proteins. These results suggest that the initial stages of synaptogenesis do not require activity. However, activity plays a critical role in the validation and stabilization of newly formed synapses. For example, blocking either NMDAR or AMPAR decreased the size of synapses, suggesting a stabilizing role for glutamate receptor activity. Either NMDAR or AMPAR blockade during development also prevented cytoskeletal remodeling, which prevents the formation of new synaptic sites. These results contrast with other experiments that suggest receptor activation plays a role in synapse elimination by noting that chronic (14 day) NMDAR blockade increased synapse number. This conflict may be due in part to the fact that the receptors are not acting alone, but in concert with other molecules like neuroligins, PSD-95, and ephrins. These proteins may produce different effects on synaptic validation in response to glutamate receptor activation [9].

One practical example of the importance of synaptic validation is the establishment of neural maps, like the topographic visual map in the thalamus and visual cortex that separates inputs from each into eye-specific columns. This map, called Ocular Dominance Columns, forms in an activity- dependent process that involves competitive neural activity. Initially, the entire visual cortex is innervated by inputs from both eyes, but due to an activity-

dependent process synapses are eliminated to form columns that receive inputs from one eye or the other. Blockade of neural activity from one eye using the sodium channel blocker TTX, or alteration of neural inputs by changing the frequency of the retinal waves from an individual eye, causes an imbalance and prevents the formation of even columns. Therefore, in this process it is not simply the presence of activity, or the total activity, but patterned activation of specific receptors that drives synaptic elimination [9].

3.2 ROLE OF THE NEUROLIGINS IN SYNAPTOGENESIS

As previously mentioned, the interaction between neuroligins and presynaptic neurexins functions to initiate synapse formation. Neuroligins are also involved in synapse validation, and this section will cover recent research on the specific role of the neuroligins on the entire process of synaptogenesis, focusing on NL1, the neuroligin variant studied in this project.

One piece of data that suggests neuroligins play a role in initiating synapse formation is the fact that both NL1 and NL2, when expressed in non-neuronal cells that are subsequently cultured with neurons, triggers synaptic formation in contacting axons. This setup, where neuronal proteins are expressed in non-neuronal cells and then cocultured with neurons to see what effect their expression has on synaptogenesis, is called the heterologous synapse system and is used as the experimental technique in this project. The details behind the assay will be discussed more thoroughly in a subsequent section. Briefly, the experimenters expressed NL1 and NL2 in HEK cells, cultured the cells with neurons, and observed synaptic vesicle clustering at the site of contact between the axon and the HEK cell. This vesicle clustering was abolished either by mutation of the cholinesterase-like domain of NL or by the addition of soluble beta-neurexin. This supports an active role of neuroligin-neurexin binding in initiating synapse formation [46].

Other experiments have supported the idea that neuroligin functions in initiating synaptic formation, and also showed these synapses are subsequently modulated by the presence of other postsynaptic proteins. Prange et al. showed that overexpression of PSD-95 results in an increase in synapse size and frequency of neurotransmitter release only at excitatory synapses, and also causes a reduced number of inhibitory synapses. Similarly, knockouts of PSD-95 increased inhibitory and decreased inhibitory synapses. In both cases, the total number of synapses remained constant. Using immunocytochemical analysis and staining synapses for glutamate or GABA, researchers showed that overexpression of all neuroligin genes results in an increase in both excitatory and inhibitory synapse number; however, simultaneous overexpression of PSD-95 and NL abolishes the increased number of inhibitory contacts. Taken together, these results suggest that the neuroligins establish synapses regardless of type, and that the eventual identity of these synapses (excitatory or inhibitory) and their subsequent maturation depends on the presence of specific post-synaptic proteins like PSD-95 [39]. This result has been seen in a variety of different brain regions, including cortex and hippocampus [6]

The different neuroligin genes appear to play different roles at excitatory and inhibitory synapses, since NL1 is found at glutamatergic synapses but NL2 is found at GABAergic synapses. Immunocytochemical and electrophysiological analysis shows that the addition of soluble beta- neurexin results in a decrease in both frequency and amplitude of postsynaptic responses at both excitatory and inhibitory synapses. This suggests that the neuroligin-beta-neurexin interaction results in maturation of excitatory and inhibitory synapses, at both the pre- and postsynapse [27].

Consistent with the specific roles for NL1 and NL2, NL1 overexpression increases the number of excitatory but not inhibitory synapses, while overexpression of NL2 increase the number of inhibitory but not excitatory synapses. Interestingly, these changes appear to be dependent on activity, since pharmacological inhibition of NMDAR prevents the increase in the number of excitatory synapses normally seen These results suggest that neuroligins

do not simply form synapses, but also specify and validate the synapse type depending on the presence of receptor activity [7].

In vivo experiments are less clear about the role of the different neuroligins in synaptogenesis. One set of experiments showed that mice lacking all three neuroligin genes (mice have three neuroligin genes compared to the five in humans) die shortly after birth due to respiratory failure. Embryonic cultures of neurons from these knockouts have both impaired inhibitory GABAergic transmission and excitatory glutamatergic transmission. These mice had substantially reduced NR1 content at their glutamatergic synapses, decreased spontaneous IPSP and EPSP frequencies, and have reduced amplitudes of evoked IPSP and EPSPs. In addition, the number of inhibitory synapses decrease while the number of excitatory synapses increase. However, the density of total synapses in the brainstem remains constant. This suggests that neuroligins are not essential for synapse formation (since synapse density remained constant), but are essential for normal synapse validation and maturation. It is not clear which cell adhesion molecule(s) substitutes for neuroligin at the knockout synapses to initiate synaptogenesis [54]. Another set of experiments, looking specifically at NL1 knockout mice, showed that the presynaptic terminals of neurons from these mice do not become stabilized, and have dynamic active zones with a limited number of NT vesicles, characteristics of immature synapses. These researchers also took neural cultures from normal mice and observed that if NL1 overexpression was induced on DIV 5, when the synapses were normally immature, then synaptic maturation occurred rapidly. Specifically, the stability of the presynaptic active zone, recruitment of presynaptic scaffolding proteins, size of the synaptic vesicle pool, and frequency of neurotransmitter release increased in neurons that overexpressed NL1. This maturation was NMDAR activity-dependent, since it was blocked by APV. Taken together, these results show that NL1 plays a role in synapse validation (specifically synapse stabilization) through an activity dependent mechanism [56]. The mechanism by which NL1 strengthens presynapses is not clear, but it appears to be a retrograde signal that involves both PSD-95 and neuroligin,

and results in increased sensitivity of the presynapse to extracellular calcium, resulting in a form of short-term plasticity [18].

In summary, the neuroligins play a role in initiating synapse formation, with NL2 functioning at inhibitory synapses while NL1 functions at excitatory synapses, and NL1 also functions to validate and stabilize excitatory synapses in an activity-dependent manner.

3.3 ROLE OF SYNCAMS IN SYNAPTOGENESIS

While less research has been done on SynCAMs compared to other adhesion molecules, these proteins appear to play a similar, but not redundant, role as neuroligins during synaptogenesis. SynCAMs initiate synapse formation, since expression of SynCAMs in non-neuronal cells followed by coculturing of these cells with neurons showed that they participate in homophilic binding and initiate synaptogenesis [3]. Also, overexpression of SynCAMs in neurons results in an increased number of synapses [16], as well as a substantial increase in the frequency of spontaneous neurotransmitter release (a measure of presynaptic strength). As previously mentioned, overexpression of NL1 shows the opposite result, with an increased number of synapses but no increase in synapse maturation [44]. More recent work suggests that SynCAMs may also specify synapse type during the formation of connections, since SynCAMs initiate the formation of excitatory, but not inhibitory, synapses [16].

Taken together, these results suggest a role of SynCAMs in both synapse initiation and synapse maturation, specifically at excitatory synapses. Whether either of these processes is activity- dependent is not known.

3.4 GLUTAMATE RECEPTOR EXPRESSION DURING DEVELOPMENT

During the activity-dependent validation process, neurotransmitter released from the presynapse provides an initial signal by acting on postsynaptic receptors. In the case of glutamatergic synapses, the composition of glutamate receptors is not constant during development; there are changes both in the type of receptor expressed and the subunit composition of these receptors. As previously mentioned, experiments studying the effects of glutamate receptor activity on the validation process using pharmacological inhibition produced conflicting results. This could be due to other molecules at the synapse, but may also be due to the type of receptor that the neurotransmitter is acting on. These changes in receptor expression could therefore have important implications in development, because activity through different receptors could potentially result in different developmental changes i.e. activity through one receptor type results in stabilization while activity through another results in refinement.

Immunocytochemical analysis found that, while both AMPARs and NMDARs are found throughout the cortex and hippocampus, there is a dramatic increase in the expression of AMPARs in the early stages of development (DIV 3 to DIV 10), while there was no change in the NMDARs expression. In fact, the proportion of synapses expressing NMDARs remained constant, at about 60 % of all synapses; the proportion of synapses expressing AMPARs increased from about 67 calcium influx through NMDARs during the course of development. It is hypothesized that NMDAR signaling early in development actually blocks the recruitment of AMPARs to the synapse, while in the adult brain NMDAR activity recruits AMPARs (this recruitment was described previously as the mechanism behind LTP) [21].

The subunit composition of the receptors change during development as well. In the forebrain and midbrain, the NMDARs switch from receptors containing the NR2B subunit

to the NR2A subunit. It is believed that the association of scaffolding proteins, like SAP102 and PSD-95, with different NMDARs results in differences in downstream signaling [53]. AMPAR undergo similar changes in subunit type. Immunocytochemistry studies have revealed consistent low levels of GluR2 and GluR4 compared to the other two AMPAR subunits. GluR1 and GluR3 both show increased transcription between DIV 11 and DIV 17 before returning to a baseline level [43]. Taken together, these results suggest that different glutamate receptor subunits are expressed at specific points during development, and this differential expression may play a critical role in allowing for specific types of postsynaptic responses at appropriate times during development.

3.5 GLUTAMATE RECEPTOR ACTIVITY AND SYNAPTIC VALIDATION

This section will provide a brief review of some recent experiments attempting to determine the role of glutamate receptor activity on synaptic validation. Almost all research in this area has focussed on the NMDARs rather than AMPARs because, as previously described, the NMDAR is expressed early in development and AMPAR is brought into the postsynaptic density as the synapse matures.

Several lines of research suggest that NMDAR activity plays a role in stabilizing synapses during development. Retinal neurons that had all NMDAR activity blocked by the application of D-APV beginning two hours after plating showed an increase in axonal branching and an increase in the speed by which axons and dendrites reshape when compared to control neurons. Interestingly, when APV was added 24 hours after plating, researchers observed no changes in the morphology of the axon, but did still see an increased number of dendritic branches. These results suggest that during development there is both activity-independent and activity-dependent stabilization. Furthermore, during activity-dependent

process, NMDAR activity stabilizes both pre- and postsynaptic structures, and NMDAR blockage results in the loss of this stability which leads to increased axon and dendrite dynamics, including an increased in dendritic branching [41].

There are several proposed mechanisms for how NMDAR stabilizes newly formed synapses. First, NMDAR activity can regulate the postsynaptic cytoskelton through the influx of calcium, which slows the turnover of actin filaments. As previously mentioned, it is also thought that NMDAR activation after the early stages of development recruits AMPARs to the postsynapse, and this recruitment not only helps increase postsynaptic responses to glutamate but also has been correlated with increased synapse size and density. Through its calcium influx, NMDAR activation may also regulate the expression of other proteins, like postsynaptic scaffolding molecules, that may promote maturation. Finally, activation of NMDAR may increase the synthesis and release of trophic factors like BDNF, which may be released into the synaptic cleft and act bidirectionally to validate formed synapses [22].

There are, however, several experiments that suggest that NMDAR activation may instead play a refining role during synapse validation. Luthi et al. took hippocampal slice cultures and administered the NMDAR blockers APV and CPP for two weeks. They found no change in postsynaptic currents, but an increase in the frequency of spontaneous neurotransmitter release. They also found that these neurons had an increased density of postsynaptic boutons, and an increase in the complexity of dendritic branching (dendritic and axonal branching typically occurs at points near where stable synapses form). Taken together, these results suggest that NMDAR activity refines newly formed synapses, which also leads to a decrease in dendritic and axonal branching [30].

Another, more recent study used selective knockouts of the NR1 subunit, and found that this deletion of NMDAR resulted in an increased number of functional synapse. This increase is blocked by the reintroduction of the NR1 subunit to the neurons that were NMDAR-deficient; this blockage is also dependent on NMDAR activity, since reintroduc-

tion of NMDAR followed by D- APV application still results in an increased number of functional synapses [1]. Additionally studies supporting the role of NMDAR in synaptic refinement found that application of APV prevented normal neural retraction that occurs during cerebellar development [40].

One final point to make is that, in this project, synaptic validation is assessed by synapse size. Several studies have found a correlation between the size of synaptic contacts and synaptic maturity and synaptic strength (measured by amplitude of spontaneous postsynaptic potentials) [51].

3.6 HETEROLOGOUS SYNAPSE SYSTEMS

The model system used in this project is called Heterologous Synapse Systems, but the terms Hemisynapses or Mixed Culture Assay are also used in the literature. This section will cover some of the important papers that have used the assay as an experimental technique.

Scheiffele et al. were the first group to discover that non-neuronal cells expressing adhesion proteins could form synapses with cocultured neurons. The group expressed both NL1 and NL2 in HEK cells and stained cocultured neurons for synapsin. They found that synapsin accumulates on axons that are in contact with NL-expressing HEK cells. They also found that staining for synaptotagmin followed by neural stimulation resulted in the exocytosis of vesicles at these heterologous synapses, indicating that the contacts are functional synapses. The group went on to show that the synapses were formed by the interaction of neuroligin and beta-neurexin, since addition of soluble beta-neurexin to the coculture or mutations to the cholinesterase-like domain of neuroligin prevented formation of these heterologous synapses [46].

Functional heterologous synapse systems were subsequently developed by Fu et al. These researchers transfected HEK cells with both NL1 and NMDAR (NR1 and NR2A or NR2B) or AMPAR (GluR2) subunits, and they found that they could record postsynaptic spontaneous currents in their heterologous synapses. These currents were equal in both amplitude and decay kinetics to neuron-neuron synapses, and the currents increased in frequency when PSD-95 was introduced into the heterologous system (mirroring in vitro overexpression studies). These results support the posit that these heterologous synapses are physiologically very similar to bona-fide neuron-neuron contacts [17].

Heterologous synapse systems were extended to include other adhesion molecules besides neuroligins. Biederer et al. showed that if the full SynCAM protein was expressed in HEK cells, and these HEK cells were cocultured with neurons, then fluorescent dyes could be used to show that vesicle exocytosis was occurring at neuron-HEK contacts. Simultaneous introduction of glutamate receptors with SynCAM also revealed spontaneous postsynaptic currents similar to both neuron-neuron synapses and the heterologous synapses formed by NL. These currents were blocked by the AMPAR antagonist CNQX as well. This paper also showed that glutamate receptors alone were not sufficient to trigger presynaptic vesicle clustering, and cocultures with HEK cells expressing only the receptors did not show spontaneous postsynaptic currents [3].

Heterologous synapses can be initiated by neuroligins and SynCAMs, but not all adhesion molecules can induce synapse formation in this assay; notable exceptions include the N-Cadherins and SALMS [10].

The initiation of heterologous synapses is not restricted to postsynaptic adhesion molecules. Nam and Chen expressed beta-neurexin in HEK cells and after coculture found PSD-95 clustering in dendrites of contacting neurons. They found that this result was specific to beta-neurexin and not other presynaptic adhesion molecules like SynCAMs and N-Cadherins, and that the synapses that were formed were primarily excitatory. Furthermore, NMDARs were clustered at these glutamatergic synapses, and AMPARs were recruited to

the contacts following glutamate application, similar to what would be expected following activity at silent synapses (see LTP subsection in background) [35].

Recent work expressing NL2 instead of NL1 in COS cells and culturing these with neurons have shown that NL2 clusters the alpha-neurexins at heterologous synapses, and that these contacts are inhibitory, GABAergic contacts. The formation of inhibitory heterologous synapses cannot be initiated by the neuroligins commonly found at excitatory synapses (NL1, 3 or 4) or by scaffolding proteins found at excitatory synapses (PSD-95). These results show that the heterologous synapse system is not specific to excitatory synapses, but can form inhibitory synapses via a molecular mechanism that is consistent with both in vitro and in vivo studies [12, 24].

While most heterologous synapse work is done in HEK cells for technical reasons (these cells are easy to transfect, and do not require trypsin to be dissociated, which limits possibility of trypsin- contamination in neural cultures), COS cells have the advantage of having a large size, which provides a greater surface area for neural contact. Chubykin et al. expressed neuroligins in COS cells and examined the heterologous synapses by labeling the presynaptic terminals for beta- neurexin and using electron microscopy. The contacts that were formed were uniform in size, and these contacts had functional presynaptic active zones with docked vesicles. Interestingly, most but not all of the contacts had electron-rich postsynaptic densities located on the non-neuronal cell [8].

There are certain instances where results from studies on heterologous synapses do not mirror traditional in vitro or in vivo studies of neuron-neuron contacts. One example is the comparison of heterologous synapses initiated by either NL1 or SynCAM. As previously mentioned, overexpression studies suggest different roles for NL1 and SynCAM during synaptogenesis; however, the electrophysiological data for heterologous synapses induced by these proteins are by all accounts identical [44].

Because of the increased use of the heterologous synapse system, a standard protocol has

been established, and was used for this project [4]. HEK cells were used in this experiment. These are from an immortal cell line originally derived from human embryonic kidney. There is some controversy surrounding the origin of these cells, since the kidney contains several different cell types [48]. However, it is important to note that these cells do not express neuronal cell adhesion molecules, receptors, or scaffolding proteins [47].

4.0 SPECIFIC AIMS

The aim of this project is to use heterologous synapse systems to

1. Compare the effect of NMDAR and AMPAR activity on synaptic validation.

2. Compare the roles of NL and SynCAM on synaptic validation

3. Examine whether expression of NMDARs or AMPARs at different times in development results in different effects on synaptic validation.

5.0 MATERIALS AND METHODS

5.1 PLASMID PREPARATION

5.1.1 Plasmids

The following plasmids were obtained for use in this project:

Table 1: Table listing all plasmids used in this project, as well as their lab source

Plasmid	**Lab Source**
pCAG-HA:NL1(ab)	Peter Scheiffele
pCl-neo-GluR1::GFP	Lu Chen
PCIS-NR1-2a:c-myc	Anne Stephenson
pcDNA1-NR2A	Jon Johnson
pCAG-SC1:FLAG	Thomas Biederer
pLL3.7-GFP and pLL3.74-dsRed	Luk Van Parijs

5.1.2 Transformation

Transformation involves introducing the plasmids of interest into E.Coli bacteria in order to prepare a greater amount of the plasmid DNA. The bacteria were incubated with the DNA, and then heat shocked to create pores in the cell to allow the DNA to enter the cell. The bacteria were then cultured overnight on media plates prepared with ampicillin; colonies that were resistant to the antibiotic had successfully incorporated the plasmid.

All plasmids were transformed into Stbl3 E. Coli cells using the following protocol. Luria Broth plates contained 100 ug/mL Ampicillin, and 25 mL of broth were in each plate.

1. Warm water bath to 42C
2. Thaw E. Coli (from -80) on wet ice
3. Add 5 ul of DNA, tap to mix
4. Incubate on ice for 30 min; place SOC media and Luria Broth plates in incubator
5. Heat shock bacteria for 45 s in water bath
6. Place on ice for 2 min
7. Add 250 ul SOC Media
8. Shake 1 h/ 37C/225 RPM
9. Plate 100 uL, incubate overnight
10. Store plates in fridge until use

5.1.3 Miniprep

A miniprep was performed in order to obtain small quantities of plasmid DNA from transfected bacteria so that a restriction digest and gel electrophoresis could be performed in order to confirm plasmid identity. Individual colonies were harvested and cultured in 3

mL liquid Luria Broth with 100 ug/mL Ampicillin. This starter culture was shaken for 12 hours at 280 rpm, and then the cells were lysed and centrifuged, and the plasmid DNA was collected from the appropriate layer.

1. Transfer 1 mL starter culture Eppe tube
2. Spin cells @ 6k/10min/4C. Remove supernatent
3. Resuspend pellet in 250 l Buffer P1 (Qiagen) and vortex
4. Add 250 l Buffer P2 (Qiagen), invert 4-6 times to mix. (Solution will turn blue).
5. Add 350 l Buffer N3 (Qiagen) and invert gently 4-6 times to mix. (Solution will turn colorless).
6. Spin 13k rpm/10min/4min. White pellet will form.
7. Transfer supernatant to spin column.
8. Centrifuge 1min/13k. Discard flow-through.
9. Add 500ul Buffer PB(Qiagen), spin 1min/13k, discard flow-through
10. Add 750ul Buffer PE(Qiagen), spin 2x for 1 min/13k, discard flow-through
11. Transfer column to sterile 1.5 mL tube, add 25uL sterile DDW, let stand 1 min.
12. Spin 1min/13k to elute DNA.
13. Optical Density reading: 4 L DNA into 80 L Total. A260=[DNA]

5.1.4 Restriction Digests

Once purified DNA was obtained, 2 ug were digested by adding the appropriate restriction enzymes and buffers. All digests were performed for 2 hours.

Table 2: Table listing all restriction digest strategies for the plasmids used in this project

Plasmid	**Restriction Enzymes**	**Number of Expected Bands**
NL1	BamH1 and EcoR1	3
NR1	EcoR1 and XBa	2
NR2A	HPa and NHE	4
GluR1	BamH1, XBa and NOT	4
pLL3.7-GFP and pLL3.74-dsRed	HindIII	5
SynCAM	EcoR1	2

5.1.5 Agarose Gel Electrophoresis

Agarose Gel Electrophoresis was performed on digested DNA in a 1.2 % gel by polymerizing agarose in TAE buffer, loading 10 uL of each sample, and running a current through the gel.

1. Pour 0.36 g of agarose into flask. Add 30mL TAE, boil for 25 seconds
2. Pour agarose/TAE with 1.5 uL EtBr into tray (with combs) when temperature is below 60C
3. Wait 20 min until gel settles
4. Take comb out, rotate gel, add the TAE until submerged
5. Add 2uL loading buffer per 10 uL sample or ladder
6. Load 10 uL of sample or ladder
7. Add 1.5 uL EtBr to both sides of gel
8. Run for 45 minutes at 115 V

9. Visualize bands under UV light

5.1.6 Midiprep and Ethanol Precipitation

Once plasmid identity is verified, milligram quantities of DNA were prepared using a standard midiprep, and purified using ethanol precipitation. Larger starter cultures (100 mL of media with 1000 uL of the miniprep starter culture) were initially prepared; the bacteria were then lysed and centrifuged, and the DNA was collected. The DNA was then purified from any protein contaminants by precipitating the plasmids out in ethanol.

1. Prepare midiculture:100 mL Luriabroth + 100 ug/mL Ampicillin + 1000 uL starter culture
2. Shake overnight at 37 C, 225 RPM
3. Harvest cells: Centrifuge 15 min/4/4200 rpm.
4. Remove supernatent
5. Resuspend in 3 mL Cell Resuspension Solution from Qiagen Midiprep Kit
6. Add 3 mL Cell Lysis Solution from Qiagen Midiprep Kit, invert gently 5 times
7. Add 6 mL neutralization solution from Qiagen Midiprep Kit, invert gently 5 times, incubate RT/ 10 min
8. Spin in ultracentrifuge with Vacuum at 4 C, 12000 RPM for 17 minutes
9. Pour supernatent into vacuum column with 10 mL resin from Qiagen Midiprep Kit, apply
10. Flush with 15 mL of 40 % isopropanol/4.2 M guanidine HCl
11. Wash with 15 mL Column Wash Solution twice
12. Spin column collectant 2 min/max speed

13. Move column to new tube, add 300 uL heated DDW, wait 2 min
14. Spin 2 min max to obtain DNA
15. Add 0.1X vol of 3 M pH 5.2 sodium acetate to samples, mix well
16. Incubate 5 min/0 C
17. Add 2.5 X vol cold 100 % ethanol, shake vigorously
18. Incubate 1 hour in freezer
19. Spin 15 min/max @ 4 C
20. Pipet out supernatent
21. Wash pellet with 1 mL ice cold 70 % ethanol
22. Aspirate out liquid, let pellet dry at 4C 1 hr
23. Resuspend in 200 uL RT DDW, leave overnight at 4C.
24. Dilute 4 uL DNA into 76 uL water, easure absorbance at 260 nm to obtain concentration of DNA
25. Dilute to 0.5 ug/ul
26. Aliquot 50 ul tubes

5.1.7 Transfection

Once plasmid DNA was prepared, the following amounts of DNA was introduced into HEK cells to create ten experimental groups.

HEK cells were transfected by adding a mixture of Lipofectamine reagent and plasmid DNA to cells bathed in media without antibiotics, incubating the cells in this mixture for three hours, and then the media was changed to remove the reagent from the cells.

Table 3: Table showing the different experimental groups prepared for this project, as well as the appropriate quantity of plasmid DNA transfected to create the groups.

Treatment Group	**DNA Amount**	**DNA Amount**	**DNA Amount**
GFP	0.8 ug pLL3.7	X	X
dsRed	0.8 ug pLL3.74	X	X
NL	0.8 ug NL	X	X
NL/AMPAR	0.4 ug NL	0.4 ug GluR1	X
NL/NMDAR	0.4 ug NL	0.2 ug NR1	0.2 ug NR2A
AMPAR	0.8 ug GluR1	X	X
NMDAR	0.4 ug NR1	0.4 ug NR2A	X
SynCAM	0.8 ug SynCAM	X	X
SynCAM/AMPAR	0.4 ug SynCAM	0.4 ug GluR1	X
SynCAM/NMDAR	0.4 ug SynCAM	0.2 ug NR1	0.2 NR2A

1. Add 400 uL/well Lam/PLL solution (10 mL DDW, 7.8 uL Lam, 23.5 uL pLL); incubate closed in hood overnight
2. Plate HEK cells at a density of 500,000 cells/mL for ICC using culture media (10 % FBS, 1
3. Allow cells to grow to 90 % confluency
4. Replace media with Transfection Media (Culture Media without antibiotics)
5. Warm OptiMem to room temperature
6. Dilute Lipofectaime (2 uL) in Optim-mem (48 uL) per well. Mix gently, incubate RT 5 min

7. Dilute DNA (0.8 ug total) in Opti-mem (50 uL total volume) per well
8. Add diluted DNA to diluted Lipofectamine
9. Mix gently, incubate 20 min RT
10. Add 100 uL to each well
11. Incubate 3 hours
12. Change media to prewarmed culture media
13. Coculture 24 hours later

5.1.8 Protein Harvest

After transfection, protein was harvested from the HEK cells in order to probe for the successful introduction of the protein coded by the plasmid using Western Blots. Cells were washed and then collected, centrifuged, resuspended in RIPA buffer, centrifuged, and finally the protein layer was collected.

Table 4: Recipe for the preparation of RIPA buffer used in the protein harvest

Ingredient	**Concentration**
Tris	20nM
NaCl	150 mM
EDTA	1 mM
Triton X-100	1%
DOC	0.5%
SDS	0.1%
PIC	1X

1. Place PBS in ice.
2. Label 1.5 mL eppe tube for each sample.
3. Make RIPA buffer (TX100 takes time to go into solution); keep on ice
4. Aspirate media, rinse 1X ice-cold PBS.
5. Add 1ml ice-cold PBS for scraping.
6. Scrape cells; transfer to 1.5 mL eppe tube.
7. Spin 3000rpm/5min/4C. (Loose pellet)
8. Carefully remove supernatant from pellet.
9. Add 50 uL RIPA buffer to each sample.
10. Pipet to mix/resuspend cells.
11. Spin 10 min/max/4C
12. Use 20uL pipet to remove 7uL + pellet (removing supernatent does not work- pulls pellet)
13. Store at -80C.

5.1.9 Western Blots

After transfection, Western Blotting was performed in order to confirm successful introduction of the protein coded for by the plasmid. The protein samples were run through a PA gel to separate the proteins by size. The proteins were then transferred to a membrane, and the membrane was then probed with antibodies for the presence of the target proteins. The antibodies were conjugated with HRP, so they could be identified by adding substrate and exposing the membrane to film.

1. Pour separating gel, overlay with water-saturated t-amyl alcohol.

Table 5: Antibodies and dilutions used for Western Blots

Treatment Group	**Antibody**	**Dilution**
NL1	Chicken Anti-HA	1:100
NR1	Mouse Anti-cMyc	1:100 to 1:2000
NR2A	Mouse Anti-NR2A	1:100 to 1:500
GluR1	Mouse Anti-GFP	1:100 to 1:1000

Table 6: Recipes for the solutions used for Western Blots

Solution	**Ingredients**
10X Running Buffer	30.3 g Tris Base; 144.2 g Glycine; 10 g SDS; 1L Total
10X Transfer Buffer	30.3 g Tris Base; 144.2 g Glycine; 1 L Total
Blotting Buffer	25 mM Tris-Cl; 0.5 M NaCl; 0.5 % Tween 20
Blocking Solution	5% w/v instant nonfat dry milk in Blotting Buffer
Antibody Solution	2% w/v instant nonfat dry milk in Blotting Buffer

2. After gel hardens, rinse top with DDW 3X, carefully dry area above gel with paper towel
3. Pour stacking gel, insert comb
4. While gel polymerizes, prepare protein samples (3 ug/uL); Denature 5 min at 95C
5. After gel hardens, remove comb, rinse wells with running buffer 3X, load into Hoefer, overlay with running buffer
6. Load 10-15uL sample/well, 10uL Ladder

7. Run 80V/gel; replenish running buffer periodically
8. Prepare Transfer buffer: 350mL DDW + 50ml 10X Transfer Buffer + 100ml MeOH
9. Prepare Transfer Case: Cathode, Plastic Grate, Sponge, Blotting Paper
10. Soak in Transfer Buffer
11. Cut gel to desired size, place blotting paper
12. Cut membrane to gel size
13. Prepare Membrane for transfer: MeOH (15sec) then DDW(2min) then rinse in transfer buffer
14. Place membrane on gel
15. Complete Transfer Case: Blotting Paper, 2 sponges, plastic grate, anode
16. Transfer 85 min, 15V
17. Block in Blocking Solution RT/2hrs on rocker
18. Add 1 Ab diluted in Ab Solution, incubate RT/1hr on rocker
19. Wash 10min 5X in Blotting Buffer
20. Add 2 Ab diluted in Ab Solution, incubate RT/1hr on rocker
21. Wash 10min 5X in Blotting buffer
22. Add SuperSignal West Pico Stable Peroxide and Luminol mixed 1:1, incubate RT/5min
23. Thoroughly aspirate chemiluminescent substrate, cover membrane in cellophane, use pipet to roll out excess substrate
24. Expose to film (start with 30s)

5.1.10 Immunocytochemistry

Immunocytochemistry was used to confirm protein expression and membrane localization. It was also used to stain heterologous synapse systems to determine the presynaptic morphology.

The antibodies used are shown in Table 7, with the dilution in parenthesis.

Table 7: Antibodies with the appropriate dilutions that were used for ICC

Protein Target	**Primary Antibody**	**Secondary Antibody**
NL1	Chicken Anti-HA (1:25)	Goat Anti-Chicken 555 (1:1000)
NR1	Mouse Anti-CMyc (1:1000)	Goat Anti-Mouse 488 or 555 (1:1000)
NR2A	Mouse Anti-NR2A (1:100)	Goat Anti-Mouse 488 or 555 (1:1000)
SynCAM	Rabbit Anti-FLAG (1:1000)	Goat Anti-Rabbit 488 or 555 (1:1000)
Synapsin	Rabbit Anti-Synapsin (1:500)	Goat Anti-Rabbit 488 or 555(1:1000)

Table 8: Recipes for the solutions used for ICC

Solution	**Ingredients**
PFA	3.8 mL DDW, 0.2 g PFA, 1X PBS, 25 uL 1 M MgCl2, 100 uL 0.5 EDTA, 0.2 g sucrose
Permeabilization Solution	0.3% Triton X-100 in PBS
Block Solution	5% BSA in PBS
Antibody Solution	1% BSA in PBS

The cells were first fixed with 4% PFA, and then permeabilized with detergent (Triton X-100). Nonspecific binding sites were then blocked with 5% BSA, and the cells were then

incubated for one hour each with first primary and then secondary antibodies. The cells were then placed onto microscope slides for visual analysis.

1. Prewarm fixative to 37C
2. Add 400uL/well prewarmed fixative to 24well dish
3. Transfer coverslip from media well to fixative well, incubate 10min
4. Appropriately discard fixative; wash PBS/5min/3X
5. Permeabilize with 500uL permeabilization solution
6. Wash PBS/5min/3X
7. Block in 500 uL blocking solution for one hour
8. Invert coverslip onto primary antibody beaded on flattened parafilm
9. Incubate 1hr
10. Return faceup to 24 well dish filled with PBS; Wash PBS 5min/3X
11. Invert coverslip onto secondary antibody beaded up on flattened parafilm
12. Incubate 1hr in dark
13. Return faceup to 24 well dish filled with PBS; Wash PBS/5min/3X
14. Submerge in Millipore water to remove PBS salts; dab excess water
15. Mount w/ 4ul Mounting Solution (Vectashied with DAPI)
16. Store 4C

5.1.11 Neural Culture

Because of IACUC restrictions, cortical neurons were harvested by other lab members from embryonic mice at day E17 and plated at a density of 500,000 cells/mL on 24 well plates

coated with laminin and poly-d-lysine using a standard protocol. Each well contained 500 uL, for a total of 250,000 cells/well. The media used consisted of Neurobasal, 2.5% GlutaMAX, 20% B27, and 10

5.1.12 Coculture

Most HEK cells were transfected with the plasmid DNA as mentioned in the Transfection Section six days after the neural harvest, and cocultured with neurons seven days after the neuron harvest. For one set of heterologous synapses with HEK cells expressing either NL or NL/NMDAR, the transfection occurred 11 days after neural harvest and the coculture occurred 12 days after harvest.

For the cocultures, HEK cells expressing the appropriate proteins were split from their original cultures and then plated onto neuronal cultures. Neuron culture media was used for all steps, and Ara-C was added to prevent HEK cells from undergoing mitosis and becoming overconfluent.

1. Equilibrate neuronal feeding medium (Neurobasal, 2.5 % GlutaMAX, 20 % B27 supplement, and 10 %)
2. Prewarm HEK culture medium and Neurobasal
3. Aspirate medium from transfected FT cells without touching well bottom
4. Wash once with PBS
5. Pipet 100uL Trypsin into center of each well, forcibly enough to dislodge cells
6. Slap 24well dish several times to dislodge cells
7. Incubate 2min
8. While incubating, firepolish filtered Pasteur pipet
9. After incubation, add Pasteur pipet full of culture medium to each well

10. Triturate transfected cells against bottom of each well until cells are separated (check under scope)
11. Collect in 15ml conical tube
12. Centrifuge 1000rpm/5min
13. Aspirate supernatant, resuspend in 5 mL PBS (must remove all Trypsin)
14. Centrifuge 1000rpm/5min
15. Resuspend pellet in 3ml equilibrated Neurobasal
16. Dilute to 4x104 cells/ml (NOT 4x106 cels/ml!)
17. Remove 250ul medium from neuronal culture (24well dish)
18. Add 250ul of transfected FT cell suspension to neuronal culture
19. Add 2 uM Ara-C to prevent HEK expansion
20. Rock gently to evenly distribute FT cells
21. Fix and stain cells either 24 hours or 48 hours after Coculture

5.1.13 Microscopy and Image Analysis

Cells were examined for protein expression and localization using a standard fluorescent microscope at 63X magnification.

Images of heterologous synapses were taken using a confocal microscope. The top and bottom of the HEK cell were determined, and fifteen slices were taken through the stack at 63X, with 1000V gain used consistently throughout all experimentation. Standard excitation bands provided by Leica were used to detect the secondary antibodies.

Images were then analyzed using ImageJ. The image analysis was carried out as follows. Images used in the explanation are from one representative heterologous synapse with the

HEK cell expressing NL1 alone. First, the confocal image stack of the synapsin staining was projected onto a single image with the maximum intensity at each pixel, as shown in Figure 9. Then, the image was thresholded to a level of 75, as shown in Figure 10; this level was chosen because it allowed for the visualization of individual contacts in the brightest images, while still allowing the analysis of the dimmer images. The number of pixels above threshold was then measured, and normalized to the cross-sectional area of the HEK cell on which the heterologous synapse was formed. This normalized total synaptic contact was then multiplied by 100 to obtain the variable that was analyzed- Percent of Total Synaptic Contact Per Cell.

A one-way ANOVA was then performed in Excel to compare Percent of Total Synaptic Contact Per Cell between treatment groups.

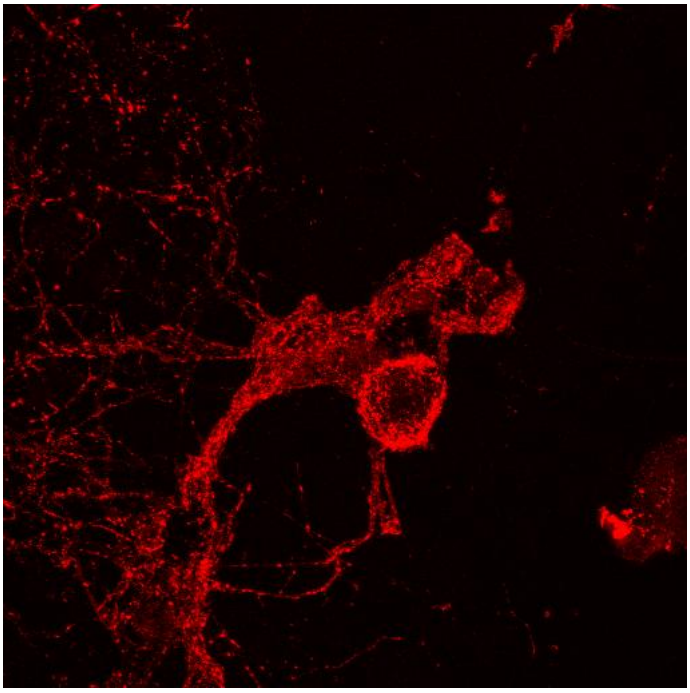


Figure 9: Image showing the projection of a confocal stack onto a single image, using maximum intensity. This heterologous synapse was formed on an HEK cell expressing Neuroligin alone, and synapsin is shown in red.

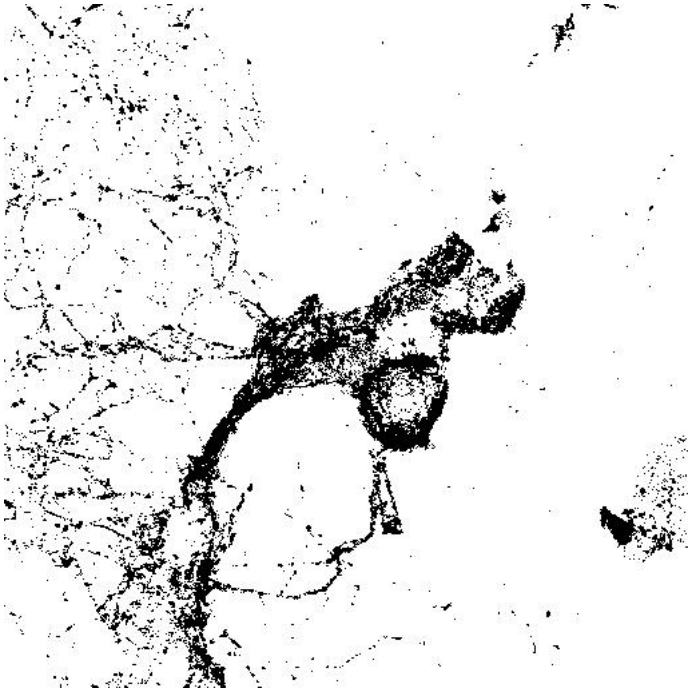


Figure 10: Image showing the thresholding of the previous image to a level of 75. This heterologous synapse was formed on an HEK cell expressing Neuroligin alone, and synapsin is shown in black.

6.0 RESULTS

6.1 CONFIRMATION OF PLASMID IDENTITY

An image of the gel electrophoresis of digested plasmids is shown below in Figure 11. This image shows the successful sequencing of all the plasmids based on the digestion strategies listed in the Restriction Digests subsection. All plasmids were digested for 2 hours; one plasmid, the pLL3.7-GFP, showed an incomplete digest using this duration; subsequent overnight digests followed by gel electrophoresis showed the appropriate fragment lengths.

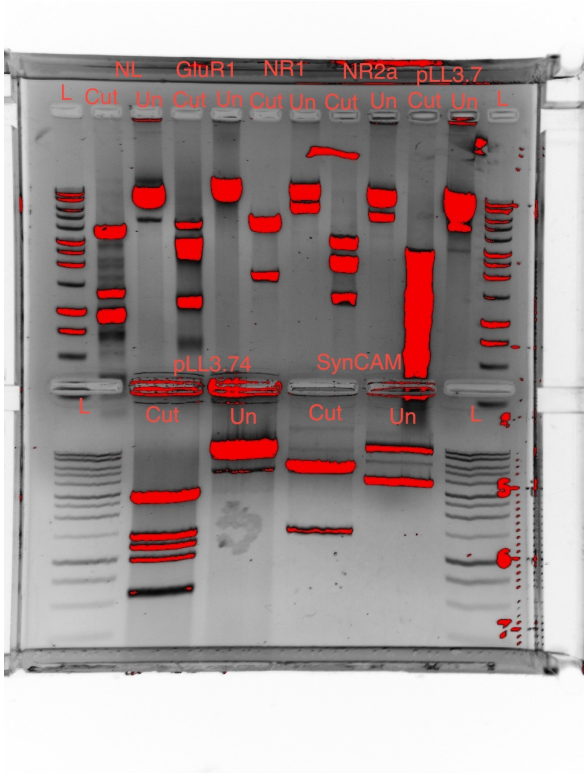


Figure 11: Image showing the results from the gel electrophoresis of digests for plasmids used. Digests were performed as discussed in the Methods section. From left to right, the lanes on the top row are: ladder; NL cut, NL uncut; NR1 cut, NR1 uncut; NR2A cut, NR2A uncut; GluR1 cut, GluR1 uncut; GFP cut, GFP uncut, and ladder. From left to right, the lanes on the bottom row are: ladder, dsRed cut, dsRed uncut; SynCAM cut, SynCAM uncut. Aside from the GFP band, which shows incomplete digestion, the bands are as expected, confirming the identity of the plasmids. The GFP was successfully sequenced following a longer, overnight digest.

6.2 HEK TREATMENT GROUPS

This section contains representative ICC images for successfully transfected HEK cells, which confirm the appropriate expression and membrane localization of the neuronal proteins that were introduced into the HEK cells.

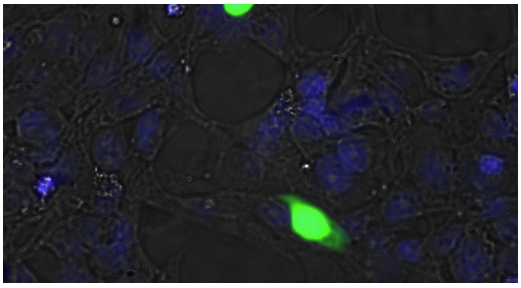
Cells that express GFP, dsRed, NL, NL/GluR1, NL/NR1/NR2A, NR1/NR2A, GluR1, SynCAM, SynCAM/AMPAR, SynCAM/NL, and non-transfected HEK cells are shown.

The image numbers, as well as the protein identified by fluorescent tag, are organized in the table below.

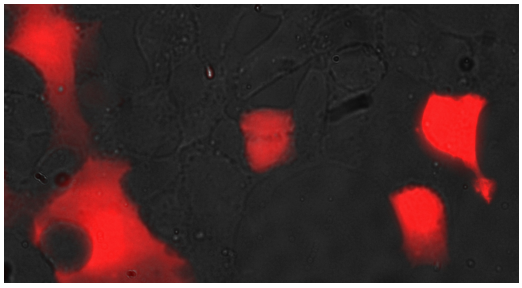
Table 9: Legend for the representative images displayed below that confirm successful transfection of HEK cells

Treatment Group	**Green**	**Red**	**Blue**	**Figure Number**
GFP	GFP		DAPI	12a
dsRed		dsRed		12b
NL		Anti-HA	DAPI	13a
NL/AMPAR	GluR1::GFP	Anti-HA	DAPI	13b
NL/NMDAR	Anti-NR2A	Anti-HA	DAPI	13c
NMDAR	X	Anti-cMyc	DAPI	14a
AMPAR	GluR1::GFP		DAPI	14b
SynCAM	Anti-FLGA		DAPI	15a
SynCAM/AMPAR	GluR1::GFP	Anti-FLAG	DAPI	15b
SynCAM/NL	Anti-FLAG	Anti-HA	DAPI	15c
Control HEKs	Anti-NR2A/cMyc/FLAG	Anti-HA	DAPI	16

In order to ensure that non-transfected HEK cells do not express the neuronal proteins of



(a) HEK cell expressing GFP



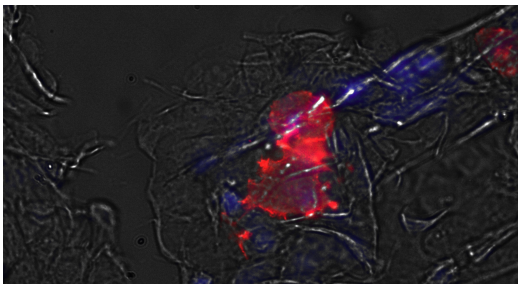
(b) HEK cell expressing dsRed

Figure 12: HEK cell transfected with either GFP (a) or dsRed (b). Nuclei are labeled blue with DAPI. Image magnification is at 63X.

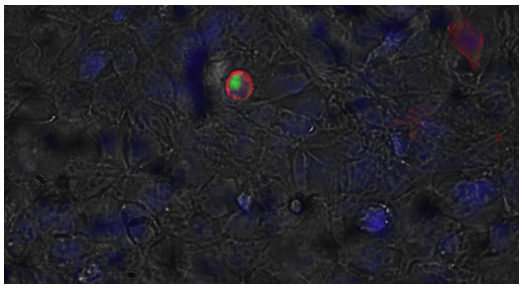
interest in this project, standard HEK cells were probed using the appropriate antibodies for NL, NR1, NR2A, and SynCAM. The result of this experiment is shown below in Figure 16.

One problem with the use of the GFP in HEK cells is that the excitation band of this protein was so broad that was excited by both the red and the green channel, producing a phenomenon where the GFP would "bleed" into the red channel. This is shown below in Figure 17.

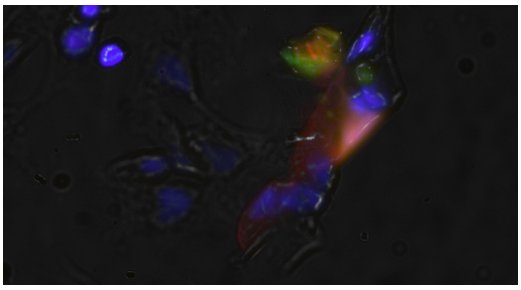
In addition to the ICC results shown above, Western Blotting was attempted in order to try and confirm proper protein expression after transfection. Only the NL blot was successful- this is shown below in Figure 18.



(a) HEK cell expressing NL

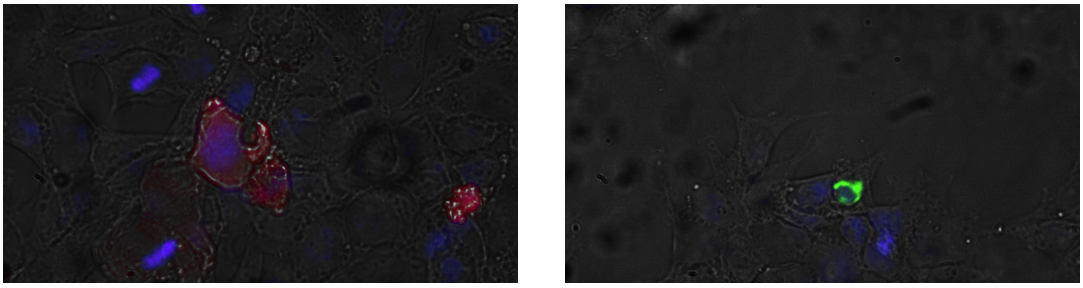


(b) HEK cell expressing NL and AMPAR



(c) HEK cell expressing NL and NMDAR

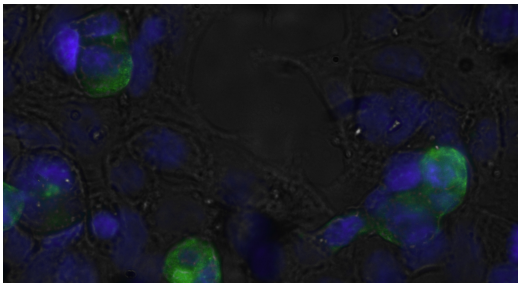
Figure 13: Representative image of HEK cells transfected with either NL, NL/AMPAR, or NL/NMDAR. NL is immuolabeled for the HA epitope (red) in (a), (b), and (c). GluR1::GFP is shown in green in (b). NR2A is immunolabeled green in (c).
All cells that expressed GluR1 or NR2A also expressed NL, but not all cells that expressed NL also expressed the receptor subunits (data not shown). Nuclei are labeled blue with DAPI. Image magnification is at 63X.



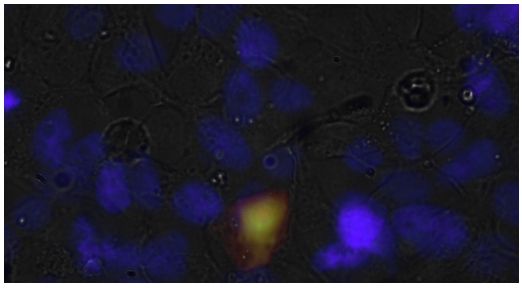
(a) HEK cell expressing NMDAR

(b) HEK cell expressing AMPAR

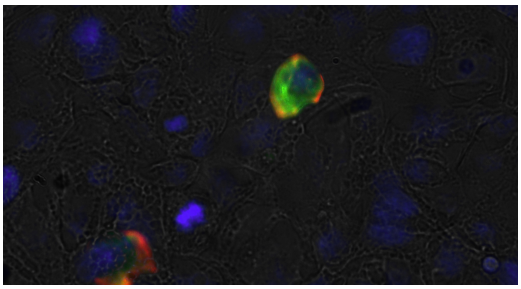
Figure 14: Representative images of HEK cells transfected with either NMDAR alone (a) or AMPAR alone (b). NR1 is immunolabeled red in (a).GluR1::GFP is shown in green in (b). Nuclei are labeled blue with DAPI. Image magnification is at 63X.



(a) HEK cell expressing SynCAM



(b) HEK cell expressing SynCAM and AMPAR



(c) HEK cell expressing SynCAM and NL

Figure 15: Representative images of HEK cells transfected with either SynCAM (a), SynCAM/AMPAR (b), or SynCAM/NL (c). NL is immuolabeled for the HA epitope (red) in (c). GluR1::GFP is shown in green in (b). SynCAM is immunolabeled for the FLAG epitope (green in (a) and (c), red in (b)). Nuclei are labeled blue with DAPI. Image magnification is at 63X.

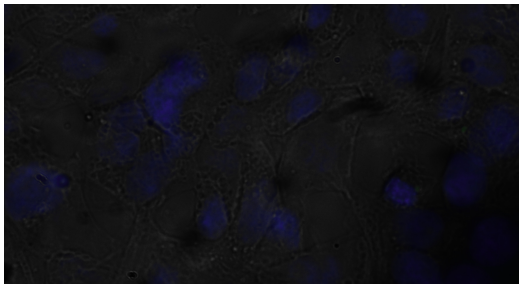


Figure 16: Image showing a nontransfected HEK cell probed for NL, NR1, NR2A, and SynCAM. Both the green and red channels are overlayed, and nuclei are labeled with DAPI (blue). Image is at 63X magnification

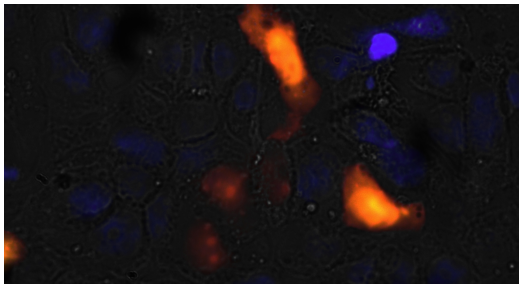


Figure 17: Image showing GFP bleeding into red channel. This is an HEK cell transfected with only GFP. The GFP is excited by both the green and the red channel, even though no protein fluorescing red is present in the cell. The response by the GFP to both channels is in green and red, and nuclei are labeled with DAPI (blue). Image is at 63X magnification

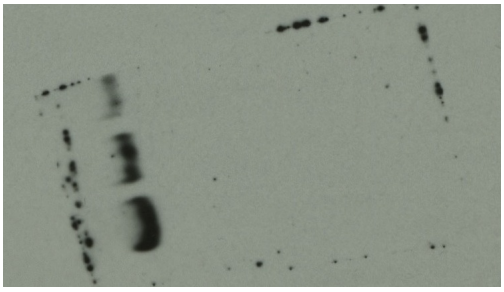


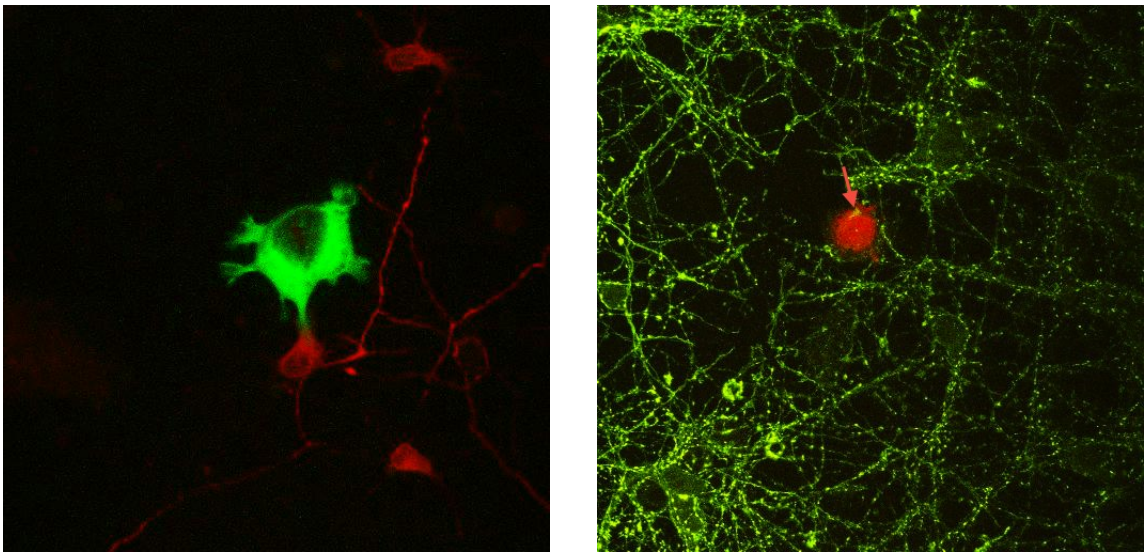
Figure 18: Image showing the Western Blot of an HEK cell transfected with NL

6.3 COCULTURE IMAGES

This section contains representative images for succesful cocultures between transfected HEK cells and neurons. These images confirm the effective formation of heterologous synapses between the neurons and HEK cells. Cocultures between neurons and HEK cells that express GFP, dsRed, NL, NL/AMPAR, NL/NMDAR, AMPAR, SynCAM/AMPAR, and SynCAM/Nl are shown below. The image numbers, as well as the protein identified by the appropriate fluorescent tag, are organized in the table below.

Table 10: Legend for the representative images shown below that confirm successful coculture between the HEK cells and neurons.

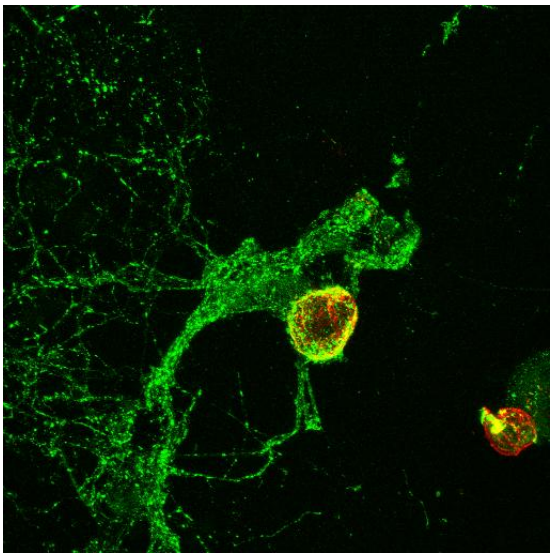
Treatment Group	**Green**	**Red**	**Figure Number**
GFP	GFP	Anti-Synapsin	19a
dsRed	Anti-Synapsin	dsRed	19b
NL	Anti-Synapsin	Anti-HA	20a
NL/AMPAR	GluR1::GFP	Anti-Synapsin	20b
NL/NMDAR	Anti-NR2A	Anti-Synapsin	20d
NMDAR	Anti-Synapsin	Anti-NR2A	21a
AMPAR	GluR1::GFP	Anti-Synapsin	21b
SynCAM/AMPAR	GluR1::GFP	Anti-Synapsin	22a
SynCAM/NL	Anti-Synapsin	Anti-HA	22b



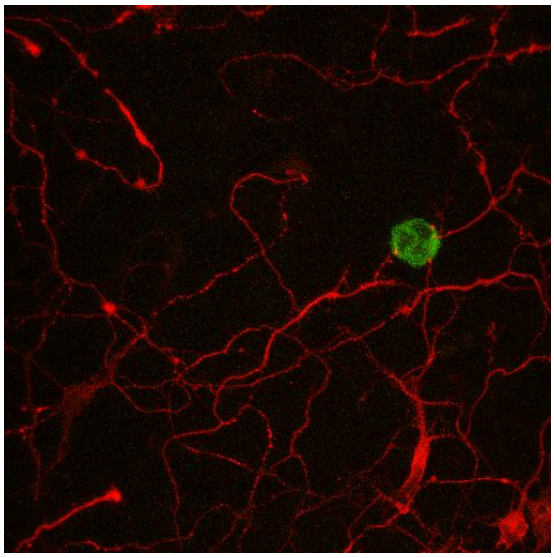
(a) Coculture between neuron and HEK cell expressing GFP

(b) Coculture between neuron and HEK cell expressing dsRed

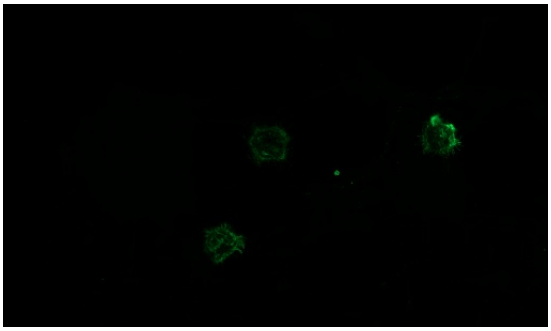
Figure 19: Representative image of a coculture between neuron and HEK cell expressing either GFP (a) or dsRed (b). GFP is green (a), and dsRed is red (b). Synapsin is immunolabeled red (a) or green (b). All HEK cells were transfected on DIV 6 and cocultured DIV 7. Image magnification is at 63X.



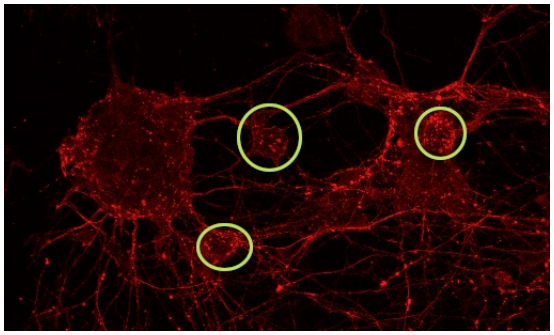
(a) Coculture between neuron and HEK cell expressing NL



(b) Coculture between neuron and HEK cell expressing NL and AMPAR

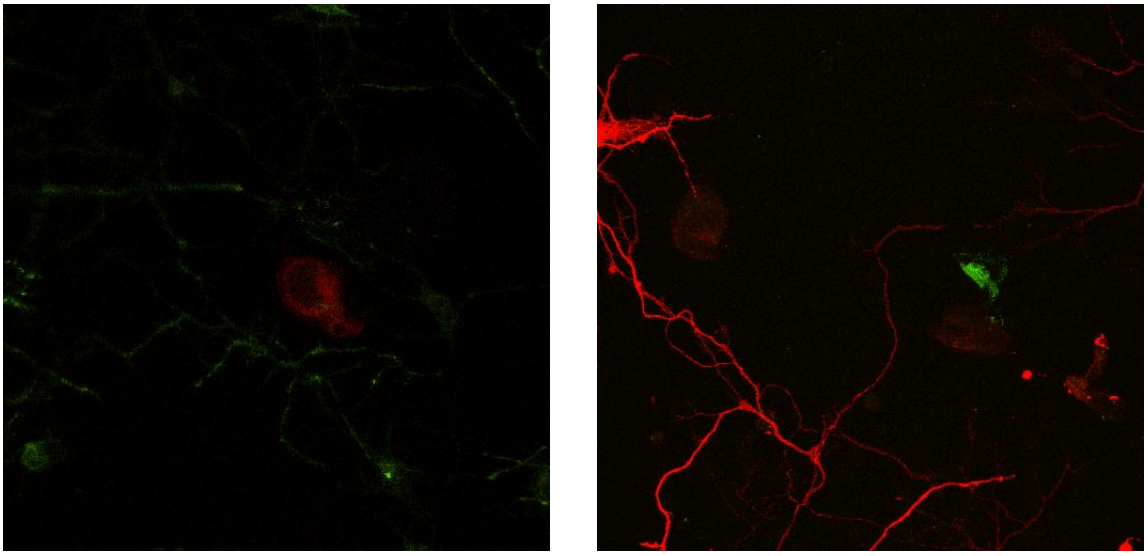


(c) Coculture between neuron and HEK cell expressing NL and NMDAR, stained for NR2a



(d) Coculture between neuron and HEK cell expressing NL and NMDAR, stained for synapsin

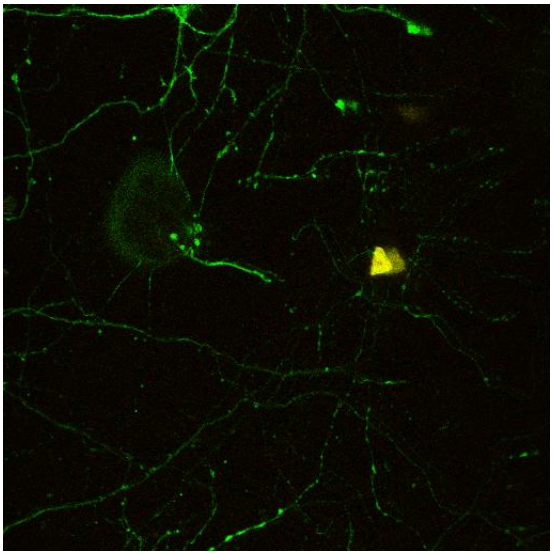
Figure 20: Representative image of a coculture between neuron and HEK cell expressing either NL (a), NL/ AMPAR (b), or NL/NMDAR (c and d). In (a), NL is immunolabeled for the HA epitope in red and synapsin is immunolabeled green. In (b), GluR::GFP is shown in green, and synapsin is immunolabeled in red. In (c), NR2A is immunolabeled green, while in (d) synapsin is immunolabeled red and the site of the HEK cells are circled. Figures (c) and (d) were separated because the NR2a antibody was too dim to be seen on an overlay. All HEK cells were transfected on DIV 6 and cocultured DIV 7. Image magnification is at 63X.



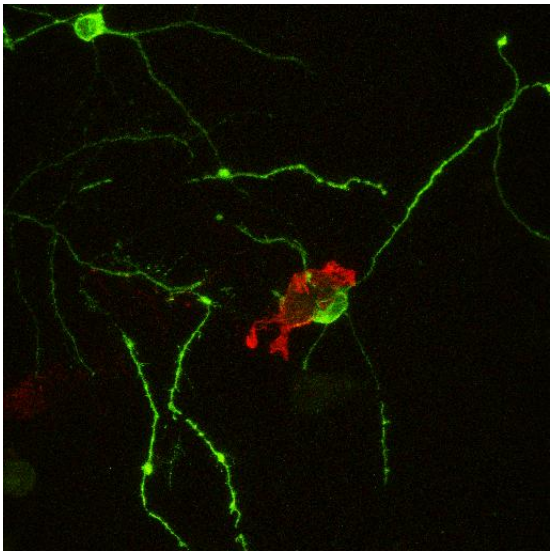
(a) Coculture between neuron and HEK cell expressing NMDAR

(b) Coculture between neuron and HEK cell expressing AMPAR

Figure 21: Representative image of a coculture between neuron and HEK cell expressing either NMDAR alone (a) or AMPAR alone (b). In (a), NR2A is immunolabeled red and synapsin is immunolabeled green. In (b), GluR1::GFP is shown in green, and synapsin is immunolabeled red. All HEK cells were transfected on DIV 6 and cocultured DIV 7. Image magnification is at 63X.



(a) Coculture between neuron and HEK cell expressing SynCAM and AMPAR



(b) Coculture between neuron and HEK cell expressing SynCAM and NL

Figure 22: Representative image of a couclutre between neuron and HEK cells expressing either SynCAM/ AMPAR (a) or SynCAM/NL (b). In (a) GluR1::GFP is shown in red, and synapsin is immunolabeled green. In (b) NL is immunolabeled for the HA epitope (red), and synapsin is immunolabeled green. All HEK cells were transfected on DIV 6 and cocultured DIV 7. Image magnification is at 63X.

6.4 COCULTURE RESULTS

For all coculture images, the presynaptic area was determined by taking the stacks of images of heterologous synapses stained for synapsin, compressing the stacks into one image with the maximum intensity at each pixel, thresholding all images, then determining the area of the synapsin staining above threshold in pixels. This area was then normalized to the area of the cross-sectional HEK cell for each heterologous synapse. The complete details of this technique, as well as figures diagramming the procedure, are shown in the Methods section.

Some samples were fixed and stained one day after coculture and others two days after coculture. When controlling for treatment groups, no statistically significant difference in presynaptic area was found between these two timepoints. These results are shown in Table 13.

Heterologous synapses between neurons and HEK cells expressing NL and either glutamate receptor were, on average, smaller than heterologous synapses where the HEK cells expressed NL alone. This difference was statistically significant when the two glutamate receptors were combined as one treatment group ($p < 0.05$), and when the NL treatment group was compared to the NL/AMPAR treatment group ($p < 0.01$) but not when the NL treatment group was compared to the NL/NMDAR treatment group ($p = 0.14$).

Heterologous synapses between neurons and HEK cells expressing SynCAM and AMPAR were, on average, much larger than those where HEK cells expressed NL and AMPAR, but the sample size was not large enough to achieve statistical significance.

Finally, a comparison of presynaptic size in HEK cells expressing NL/NMDAR that were cocultured 12 days after neural harvest compared to 7 days after harvest shows that synapse size is larger in those heterologous synapses cocultured later, and this difference is statistically significant. These results are summarized in Table 13.

A table showing each treatment, with number of cocultures, average presynaptic size and standard deviation, and the results of the T-Test comparing the various treatment groups to the NL group is shown below in Table 11. In this table, SC is an abbreviation for SynCAM.

A table specifically comparing the heterologous synapses for HEK cells expressing NL/AMPAR and those expressing SynCAM/AMPAR is shown in Table 12.

Table 11: Summary of Results

Treatment Group	**NL**	**NL/AMPAR**	**NL/NMDAR**	**SC/AMPAR**	**SC/ NL**
Number of Cells	26	6	8	3	2
Average Total Synaptic Area, %	65	45	51	67	19
Standard Deviation	33	10	20	56	1.0
P-Value (compared to NL)		0.01	0.14	0.64	

Table 12: Neuroligin vs SynCAM Results

Treatment Group	**NL/AMPAR**	**SynCAM/AMPAR**
Number of Cells	6	3
Average Total Synaptic Area, %	45	84
Standard Deviation	10	60
P-Value		0.38

Table 13: Comparison of Timepoint Results

Treatment Group	**NL/NMDAR, DIV 7**	**NL/NMDAR, DIV 12**
Number of Cells	4	4
Average Total Synaptic Area, %	64	39
Standard Deviation	14	13
P-Value		0.02

7.0 DISCUSSION AND FUTURE DIRECTIONS

7.1 EXPRESSION OF NEURONAL PROTEINS BY HEK CELLS

The digest of plasmid DNA followed by gel electrophoresis and sequencing was an important first step in this project. None of the plasmids were manufactured in our lab, and all had their sequences confirmed by the research groups that created them. However, at every stage of the purification process there is the potential for contamination. As seen in Figure 11, the gel shows the expected bands for the desired plasmids.

The plasmids were then transfected into HEK cells to create the various experimental groups. Originally, calcium phosphate transfection was used to introduce the DNA. However, this had a poor transfection efficiency. Since the possibility of heterologous synapse formation depends not only on the density of HEK cells and neurons in the culture, but also on the successful expression of neuronal proteins by the HEK cell, Lipofectamine transfection was utilized throughout the remainder of the project.

Initially, Western Blotting was used to confirm protein identification. However, only blots probing for NL were successful. Blots probing for all other proteins showed a blank film with nothing labeled. Some of the possible explanations are described below.

First, the NR2A subunit has a large molecular weight (180 kDa, compared to 115 kDa for NL). This could result in problems during the transfer of the protein from the gel to

the membrane. Because the process involves placing the membrane over the gel and then applying a voltage to pull the protein out of the gel, larger proteins move more slowly, and some may not be able to move through the pores during the transfer time. To see whether this was the issue, two different parameters were varied. First, the voltage applied during the transfer was increased from 15V to 30V in order to see whether providing an increased electrostatic force could pull the larger proteins through without denaturing. Second, the duration of the transfer was increased from 75 minutes to 120 minutes in order to see whether a longer application of the voltage could pull the larger proteins onto the membrane without also pulling smaller proteins off of the membrane and into solution. Neither of these adapted transfer processes resulted in successful probing for the transfected proteins.

The second potential reason for why the Western Blots were not successful may be because, as mentioned in the Methods Section, several of the proteins were probed using epitope markers. These are short peptide fragments that are artificially introduced into the protein by adding the appropriate nucleotides to the gene sequence (similar to fluorescent tags). They do not alter protein function, but can be used to identify transfected, and not endogenous, proteins. It was important to use epitope tagging in this project because all the proteins that were introduced into the HEK cells are already expressed by neurons. If cocultures were stained simply for the neuronal proteins, it would be difficult to separate the immunocytochemical staining of transfected protein from the neuronal protein, and this would complicate the image analysis of the heterologous synapses. Using epitope tags avoids this issue. The issue with using these tags is that they are designed to be identified using immunocytochemical detection. It is possible that while running the gel to separate proteins, or during the transfer process, the tag is made unavailable for detection by antibodies.

Despite the issues with using Western Blots to confirm protein expression, immunocytochemistry shows that these plasmids were successfully transfected into the HEK cells.

These images also showed that the proteins were primarily localized to the membrane. This is an important criterion, since the heterologous synapses occur between the axon and the membrane of the HEK cell, and so the proteins have to be located on the cell surface in order to interact with the contacting neuron.

All treatment groups had some HEK cells that expressed the plasmids, but the expression efficiency was not consistent between the different proteins. Qualitatively, the same number of cells were successfully transfected with GFP, dsRed, NL, or NR. However, relatively fewer cells transfected with GluR1 or NR2A expressed these proteins. This could be due to a variety of issues, including the efficiency by which the plasmid is introduced into the cell, or the ability of the cell to transcribe the plasmid. This limitation was overcome by identifying transfected cells by the presence of GluR1 or NR2A, since for the NL/AMPAR and NL/NMDAR treatment groups, all cells that expressed NL also expressed GluR1 or NR2A, but not all cells that expressed GluR1 or NR2A also expressed NL.

One final point to note about the transfection of the various treatment groups is the HEK cells expressing GFP show bright fluorescence under both the red and green channels of the microscope. This causes problems when these cells are cocultured with neurons, since it is difficult to differentiate whether the red is from the GFP or from the labeled synapsin protein on the neurons. Since the intensity of fluorescence was relatively equal through both the green and the red channel, this was overcome by subtracting the green channel intensity from the red channel intensity when analyzing the cocultures for synapsin area. Images of the red channel of a coculture before and after this subtraction are shown below in Figures 23 and 24.

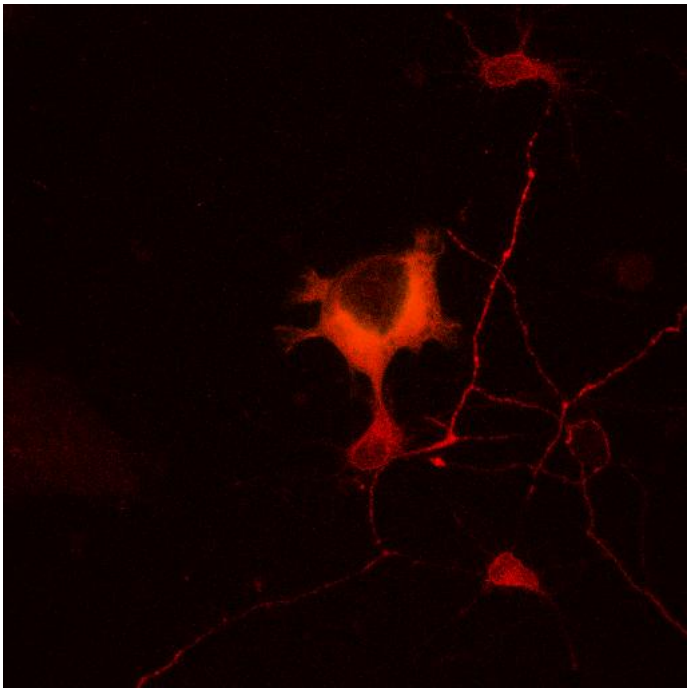


Figure 23: Image showing the successful coculture of a neuron with an HEK cell expressing GFP. Synapsin is labeled red, but there is bleedthrough from the green into the red channel.

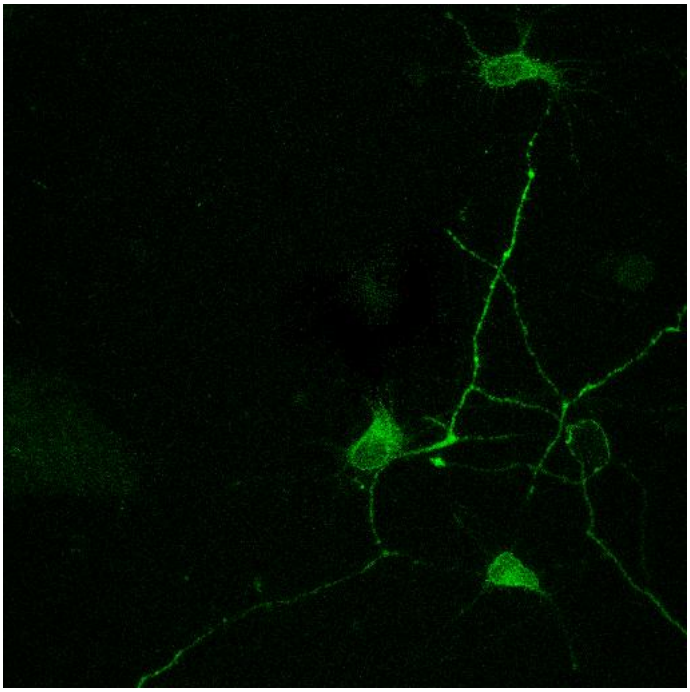


Figure 24: Image showing the subtraction of the green channel from the red channel. Synapsin is shown in green.

7.2 DISCUSSION OF COCULTURE RESULTS

In this project, the presynaptic size in heterologous synapses was measured by staining the contacting axons for VAMP, synaptotagmin, and synapsin. However, the antibodies for VAMP and synaptotagmin did not prove to be as successful as synapsin in producing individual puncta at synaptic sites. The antibody against VAMP, even at a dilution of 1:100, did not label synapses brightly enough. The synaptotagmin antibody, even at a dilution of 1:2000, lit up the entire neuronal process extremely brightly, as seen in Figure 25. For this reason, all analysis focussed on synapsin staining. Future work should also try to correlate the synapsin results with stainings for other presynaptic proteins.

It is important to note that the results from this analysis are complicated by three factors. First, there is inherent randomness to the formation of heterologous synapses. Since the cultures are not controlled, neurons can grow their processes in any direction, and so they may miss coming into contact with cocultured HEK cells. The HEK cells that the neurons do come into contact with also need to be expressing the appropriate postsynaptic proteins, and because of the poor expression of GluR1 and NR2A, neurons formed more heterologous synapses with HEK cells expressing NL alone than NL/AMPAR and NL/NMDAR. When the HEK cells are seeded on neurons in a 24 well dish, a single well typically yields anywhere from 0 to 5 heterologous contacts. Finally, the antibody used to stain SynCAM was developed in a rabbit host, the same as synapsin. Because of this, it was not possible to do immunocytochemical analysis on heterologous synapses with HEK cells expressing SynCAM alone.

There was no accumulation of synapsin in contacting axons of neurons cocultured with HEK cells expressing GFP, dsRed, NMDAR, or AMPAR. This is expected, since HEK cells do not, on their own, express the adhesion molecules that are necessary to initiate synapse formation. The expression of the glutamate receptors alone also is not sufficient to cause synaptogenesis to occur. There are some interesting qualitative observations from

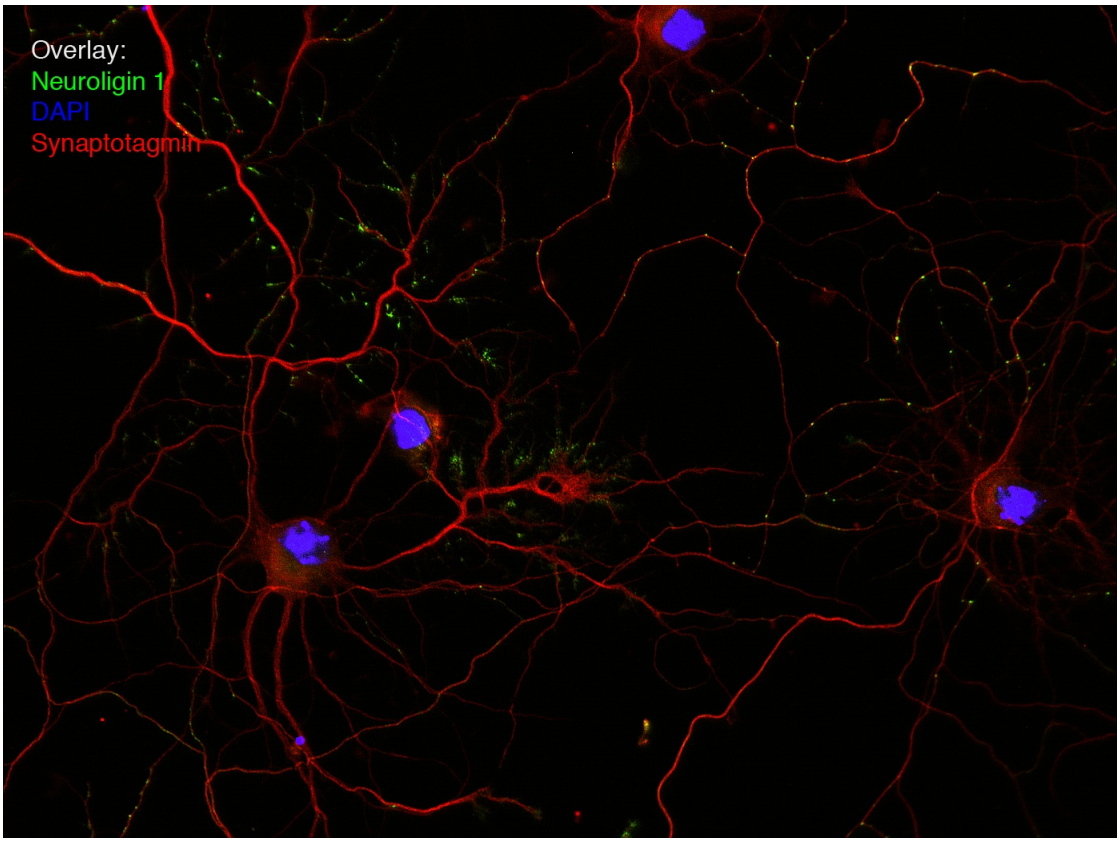


Figure 25: Image showing a neuron culture stained for synaptotagmin and neuroligin. The important thing to note is that the synaptotagmin staining lights up the entire process brightly, even after diluting the primary antibody by 1:2000. This makes it difficult to identify individual sites of synaptic contact.

images of these cocultures. First, it was difficult to determine the difference between synapsin in contacting axons and GFP in HEK cells because of excitation of GFP by the red channel, which made it difficult to differentiate red fluorescent proteins from green fluorescent proteins in cocultures. For this reason, the expression of a red fluorescent protein served as the primary control. In the image of this coculture (Figure 26), some points of contact between the axon and the HEK cell can be seen. These contacts do not disappear when the image is thresholded (Figure 27), but the areas are much smaller than the sites of contact formed with HEK cells expressing NL. It can be hypothesized that the axon recognizes the surface as the HEK cell as a potential site for synaptogenesis, but does not initiate synapse formation and synapsin localization because the appropriate cell adhesion molecules are not present.

The analysis of the amount of synapsin staining in contacting axons shows a trend where the expression of glutamate receptors results in a smaller presynaptic area than in heterologous synapses expressing NL alone. This difference was statistically significant between the NL and the NL/AMPAR treatment groups, but not for the NL and the NL/NMDAR treatment groups.

It is also interesting to note that in all cases, the postsynaptic proteins that were transfected into the HEK cell did not target to the heterologous synapse, but instead remained localized around the entire membrane of the cell. As mentioned in the Theory Section, NMDAR and AMPAR targeting to the synapse is mediated by other postsynaptic proteins, like PSD-95, NARP, Syndecan, and the EphB Receptor. Since these proteins are absent in the HEK cells, it is not unusual to expect that these proteins are not localized to the neuron contact. The targeting of NL1 is slightly more complicated, since it is hypothesized that PSD-95 is involved in the recruitment of these proteins to the synapse, but mutations to the PDZ domain on PSD-95 that binds to NL do not result in alterations in its synaptic targeting. Future work may involve introducing NARP, Syndecan, or the EphB receptors into HEK cells and examining whether the NMDAR and AMPAR are then localized to the

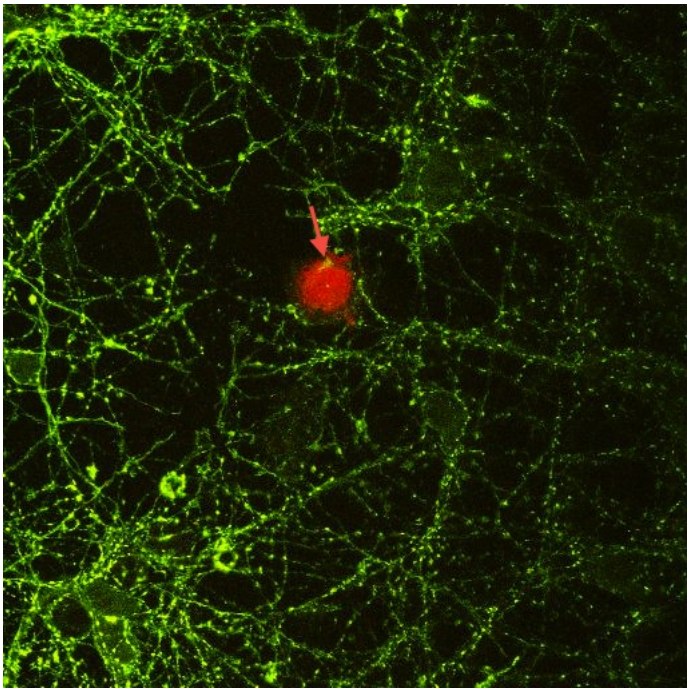


Figure 26: Image showing the successful coculture of a neuron with an HEK cell expressing dsRed. dsRed is red, and synapsin is labeled green. Arrows label sites of potential contact Image is from a confocal micrscope at 40X magnification

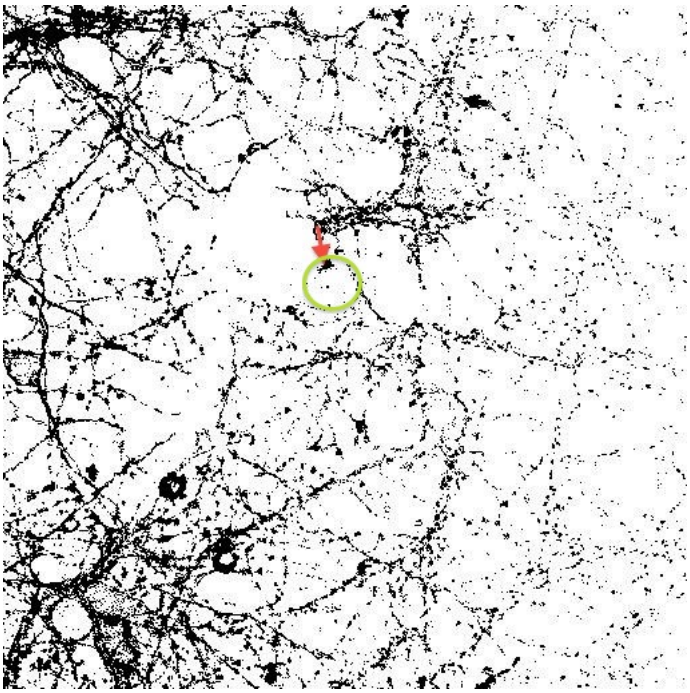


Figure 27: Image showing the successful coculture of a neuron with an HEK cell expressing dsRed, after threshold. Black indicates synapsin. Arrows label sites of potential contact, and the location of the original HEK cell is circled in green. Image is from a confocal micrscope at 40X magnification

synapse. It may also prove interesting to see whether the localization of these receptors to the synapse results in any changes in synapse validation, since the glutamate receptors are now directly opposed to the presynaptic release machinery. This system can also be used as an assay to screen proteins that are potentially involved in synaptic targeting of NL1 by introducing candidate proteins into the HEK cell and examining heterologous synapses for NL1 targeting.

Because of the prevalence of NMDAR at early synapses, few experiments have examined the role of AMPAR activity on activity-dependent validation. The results of this project suggest that AMPAR activity serves to refine initial synaptic contacts. The trend in heterologous synapse size between the NL treatment group and the NL/NMDAR treatment group are consistent with the results produced by Luthi et al [30], which found that long-term administration of NMDAR blocker resulted in an increased density of synaptic contacts. The absence of NMDAR expression can mirror long-term pharmacological inhibition, since the receptors are not present at the postsynapse to decipher neurotransmitter release. These results support the hypothesis that NMDAR activation results in synaptic refinement.

Other studies have found evidence supporting the view that glutamate receptor activity results in synaptic stabilization. In particular, calcium influx through the NMDAR is thought to stabilize synapses by regulating the cytosekelton, recruiting AMPAR, regulating gene expression, and increasing the synthesis of trophic factors. Furthermore, experiments on the role of NL in validating initial excitatory contacts was found to be dependent on NMDAR activity. It could be that other postsynaptic proteins could be contributing to produce this net effect. In particular, PSD-95 has been found to be a critical role in the validation of excitatory synapses. The absence of PSD-95 (and the other postsynaptic proteins that this scaffolding molecule complexes at the synapse) in these heterologous synapses could be one reason for the discrepancy between the results of this project and the other experiments studying excitatory synapse validation.

Several experiments have found a role for NL1 in validating excitatory synapses in a manner that is dependent on NMDAR activation. The results of this project do not reflect this hypothesis, since the NL/NMDAR treatment group had heterologous synapses that were smaller than the NL treatment group (though the number of replicates did not allow these results to achieve statistical significance). These experiments can be reconciled with the results of this project by recognizing that in heterologous synapses, nearly all postsynaptic constituents are absent. It is possible that the stabilization of newly formed synapses requires calcium influx through the NMDAR that then acts upon another protein (e.g. Protein Kinase C, or CaMKII) which is not present in this system. In this sense, NMDAR activation alone is not sufficient to trigger the stabilization that is seen in these other experiments.

Because of the differential expression of glutamate receptors during development, it is possible that more mature neurons may be responding differently to the retrograde signal from the postsynapse that result from NMDAR activation. This hypothesis was tested by coculturing neurons at a later time point (DIV 12 compared to DIV 7) and then comparing presynaptic size within a treatment group. There was only enough time to complete experimental groups containing NL/NMDAR. An analysis of the synapsin staining shows that cocultures with more mature neurons resulted in smaller heterologous synapses, and that this difference was statistically significant. This result suggests that as neurons become more developmentally mature, they respond differently to retrograde signals resulting from postsynaptic NMDAR activity; NMDAR activity seems to play a greater role in refinement in older cultures. Successful cocultures for NL and NL/AMPAR are needed at DIV 12 in order to examine how the absence of glutamate activity, and how AMPAR activity, changes as neurons mature.

A comparison of heterologous synapses between neurons and HEK cells expressing NL and HEK cells expressing SynCAM is limited here because of the small number of data points, and the lack of successful heterologous synapses between neurons and HEK cells

expressing SynCAM/ NMDAR. The average presynaptic area is larger in cells expressing SynCAM/AMPAR compared to NL/AMPAR, but the error, particularly in the SynCAM/AMPAR group, is almost as large as the average value and the results are not statistically significant. More cocultures with HEK cells expressing SynCAM/AMPAR and SynCAM/NMDAR need to be performed before any conclusions can be drawn. Interestingly, a comparision of heterologous synapses between HEK cells with NL and HEK cells with NL/SynCAM show that NL/SynCAM has a much smaller presynaptic size, with a very low p-value ($p< 0.001$). However, this is most likely due to the small deviation in presynaptic size in the NL/SynCAM treatment group, and the small number of synapses found in this group ($n = 2$) suggests that more experiments need to be performed to identify a greater number of heterologous synapses before a conclusion can be drawn.

It is important to emphasize that heterologous synapses are an inherently simplified system. Because many aspects of the postsynapse, including different proteins, dendritic outgrowth, and cytoskeletal structures, are absent in this technique, the results of this project needs to be used in conjunction with bona-fide neuron-neuron experimentation in order to understand the molecular dynamics of synaptogenesis.

7.3 FUTURE DIRECTIONS

The next step in this project is to obtain heterologous synapses from the remaining experimental groups. This would include more data points for the SynCAM, SynCAM/AMPAR and SynCAM/NL treatments, in order to determine the activity-dependence of synaptic validatino by SynCAM. In order to study the time course of development, cocultures with mature neurons at DIV 12 and HEK cells expressing NL and NL/AMPAR will be obtained as well. If significant differences in the size of heterologous synapses between these time points are found, more thorough time courses can be obtained.

The inherent randomness of heterologous synapse formation was discussed in the last section. One future direction for this project could then be to coculture neurons and HEK cells in a microfluidic device that guides axon outgrowth from the neuron to the transfected HEK cell. This could be accomplished by culturing the neurons in one chamber and the HEK cells in another. Strips between the two chambers could then be coated with molecules like laminin and poly-D- lysine to promote axonal outgrowth. This directed outgrowth would result in a greater number of heterologous synapses for a given experimental group.

It would also be useful to introduce more proteins into the HEK cell in order to study how postsynaptic interactions affect synaptic validation. For example, the scaffolding proteins PSD-95 and Stargazin can be included along with NL and the glutamate receptors to see how complexing the receptors to the cell adhesion molecule affects synaptic development. Other postsynaptic proteins involved in synaptogenesis, like CaMKII, could also be introduced. It would also be interesting to introduce NL and both NMDAR and AMPAR into the same HEK cell, and see how combined expression of two different glutamate receptors affects synaptic validation. It may also prove useful to introduce the proteins involved in glutamate receptor targeting at the synapse (e.g. Syndecan, NARP, or EphB Receptor) to see what effects these proteins have individually and collectively on not only targeting but also synaptic validation.

The limitation with the techniques of this project is that Lipofectamine transfection is only capable of introducing up to three plasmids into a cell. Other gene delivery techniques, like Lentivirus delivery, would have to be used to pursue these other types of experiment.

7.4 ACKNOWELDGEMENTS

I would like to acknowledge

- Dr. Henry Zeringue for mentorship and project guidance
- Shawn Burton, Ashwin Vishwanathan, and Dr. Ed Plowey for neuronal cultures
- The Davidson Lab at the University of Pittsburgh for use of confocal microscope
- Dr. Jon Johnson, Dr. Sanjeev Shroff, and Dr. Gong Chen for serving on the thesis committee
- The University Honors College at the University of Pittsburgh for project funding and the opportunity to pursue this unique degree program

BIBLIOGRAPHY

[1] Hilel Adesnik, Guangnan Li, Matthew J During, Samuel Pleasure, and Roger Nicoll.
Nmda receptors inhibit synapse unsilencing during brain development.
Proceedings of the National Academy of Sciences, 105:5597–5602, 2008.

[2] Hafiz Ahmed.
Principles and Reactions of Protein Extraction, Purification and Characterization.
Taylor and Francis, London UK, 2005.

[3] Thomas Biederer, Yildirim Sara, Marina Mozhayeva, Deniz Atasoy, Xinran Liu, Ege Kavalali, and Thomas Sudhof.
Syncam, a synaptic adhesion molecule that drive synaptic assembly.
Science, 297:1525–1530, 2002.

[4] Thomas Biederer and Peter Scheiffele.
Mixed-culture assays for analyzing neuronal synapse formation.
Nature Protocols, 2:670–676, 2007.

[5] W. Neal Burnette.
Western blotting: Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein a.
Analytical Biochemistry, 112:195–203, 1981.

[6] Ben Chih, Holly Engelman, and Peter Scheiffele.
Control of excitatory and inhibitory synapse formation by neuroligins.
Science, 307:1324–1328, 2005.

[7] Alexander Chubykin, Deniz Atasoy, Mark Etherton, Nils Brose, Ege Kavalali, Jay Gibson, and Thomas Sudhof.
Activity-dependent validation of excitatory versus inhibitory synapses by neuroligin-1 versus neuroligin-2.

Neuron, 54:919–931, 2007.

[8] Alexander Chubykin, Xinran Liu, Davide Comoletti, Igor Tsigelny, Palmer Taylor, and Thomas Sudhof.
Dissection of synapse inductions by neuroligins.
Journal of Biological Chemistry, 280:22365–22374, 2005.

[9] Susana Cohen-Cory.
The developing synapse: Construction and modulation of synaptic structures and circuits.
Science, 298:770–776, 2002.

[10] Matthew Dalva, Andrew McClelland, and Matthew Kayser.
Cell adhesion molecules: signalling functions at the synapse.
Nature Neuroscience, 8:206–220, 2007.

[11] Raymond Dingledine, Karin Borges, Derek Bowie, and Stephen F. Traynelis.
The glutamate receptor ion channel.
Pharmacological Reviews, 51:7–61, 1999.

[12] Ning Dong, Jinshun Qi, and Gong Chen.
Molecular reconstitution of functional gabaergic synapses with expression of neuroligin-2 and gabaa receptors.
Molecular and Cellular Neuroscience, 35:14–23, 2007.

[13] Thomas Dresbach, Antje Neeb, Guido Meyer, Eckart Gundelfinger, and Nils Brose.
Synaptic targeting of neuroligin is independent of neurexin and sap90/psd95 binding.
Molecular and Cellular Neuroscience, 27:227–235, 2004.

[14] B. Edmonds, AJ Gibb, and D Colquhoun.
Mechanisms of activation of glutamate receptors and the time course of excitatory synaptic currents.
Annual Revue of Physiology, 57:495–519, 1995.

[15] Philip Felgner, Thomas Gadek, Marilyn Holm, Richard Roman, Hardy Chan, Michael Wenz, Jeffrey Northrop, Gordon Ringold, and Mark Danielsen.
Lipofection: A highly efficient, lipid-mediated dna transfection procedure.
Proceedings of the National Academy of Science, 84:7413–7417, 1987.

[16] Adam Fogel, Michael Akins, Alexander Krupp, Massimiliano Stagi, Valentin Stein, and Thomas Biederer.
Syncams organize synapses through heterophilic adhesion.
Journal of Neuorscience, 27:12516–12530, 2007.

[17] Zhanyan Fu, Phillip Washbourne, Pavel Ortinski, and Stefano Vicini.
Functional excitatory synapses in hek293 cells expressing neuroligin and glutamate receptors.
Journal of Neurophysiology, 90:3950–3957, 2003.

[18] Kensuke Futai, Myung Jong Kim, Tsutomu Hasikawa, Peter Scheiffele, Morgan Sheng, and Yasunori Hayashi.
Retrograde modulation of presynaptic release probability through signaling mediated by psd-95-neuroligin.
Nature Neuroscience, 10:186–195, 2007.

[19] Craig Garner, Grace Zhai, Eckart Gundelfinger, and Noam Ziv.
Molecular mechanisms of cns synaptogenesis.
Trends in Neuroscience, 25:243–250, 2002.

[20] Craig C Garner, Stefan Kindler, and Eckart D Gundelfinger.
Molecular determinants of presynaptic active zones.
Current Opinion in Neurobiology, 10:321–327, 2000.

[21] Ben Hall and Anirvan Ghosh.
Regulation of ampa receptor recruitment at developing synapses.
Trends in Neuroscience, 31:82–89, 2007.

[22] Jackie Hua and Stephen Smith.
Neural activity and the dynamics of central nervous system development.
Nature Neuroscience, 7:327–332, 2004.

[23] P. Jonas and N. Burnashev.
Molecular mechanisms controlling calcium entry through ampa-type glutamate receptor channels.
Neuron, 15:987–990, 1995.

[24] Yunhee Kang, XueZhao Zhang, Frederick Dobie, Huaiyang Wu, and Ann Marie Craig.
Induction of gabaergic postsynaptic differentiation by alpha-neurexins.
Journal of Biological Chemistry, 283:2323–2334, 2007.

[25] Eunjoon Kim and Morgan Sheng.
Pdz domain proteins of synapses.
Nature Neuroscience, 771:771–781, 2004.

[26] Jee Hae Kim and Richard L Huganir.
Organization and regulation of proteins at synapses.
Current Opinion in Cell Biology, 11:248–254, 1999.

[27] Joshua Levinson, Nadege Chery, Kun Huang, Tak Pan Wong, Kimberly Gerrow, Rujun Kang, Oliver Prange, Yu Tian Wang, and Alaa El-Husseini.
Neuroligins mediate excitatory and inhibitory synapse formation.
Journal of Biological Chemistry, 280:17312–17319, 2005.

[28] Gary Lipps.
Plasmids: Current Research and Future Trends.
Caister Academic Press, Norfolk UK, 2008.

[29] M-F Lise and A. El-Husseini.
The neuroligin and neurexin families: from structure to function at the synapse.
Cell and Molecular Life Sciences, 63:1833–1849, 2006.

[30] Andreas Luthi, Lucia Schwyzer, Jose Maria Mateos, Beat Gahwiler, and Anne McKinney.
Nmda receptor activation limits the number of synaptic connections during hippocampal development.
Nature, 4:1102–1107, 2001.

[31] Gary G. Matthews.
Neurobiology: Molecules, Cells, and Systems- 2nd Edition.
Blackwell Science Ltd, Malden, Massachusetts, 2000.

[32] Gary G. Matthews.
Cellular Physiology of Nerve and Muscle- 4th Edition.
Blackwell Science Ltd, Malden, Massachusetts, 2003.

[33] Mark L Mayer.
Glutamate receptor ion channels.
Current Opinions in Neurobiology, 15:282–288, 2005.

[34] Martine Migaud, Paul Charlesworth, Maureen Dempster, Lorna Webster, Ayako Watabe, Michael Makhinson, Yong He, Mark Ramsay, Richard Morris, John Morrison, Thomas Dell, and Seth Grant.
Enhanced longterm potentiation in impaired earning in mice with mutant postsynaptic density 95 protein.
Nature, 396:433–439, 1998.

[35] Christine Nam and Lu Chen.
Postsynaptic assembly induced by neurexin-neuroligin interaction and neurotransmitter.
Proceedings of the National Academy of Science, 102:6137–6142, 2005.

[36] John G. Nicholls, A. Robert Martin, Bruce G. Wallace, and Paul A. Fuchs.
From Neuron To Brain- 4th Edition.
Sinauer Associates, Inc., Sunderland, Massachusetts, 2001.

[37] Seiji Ozawa, Haruyuki Kamiya, and Keisuke Tsuzuki.
Glutamate receptors in the mammalian central nervous system.
Progress in Neurobiology, 54:581–618, 1998.

[38] Maksymilian Pluta.
Advanced Light Microscopy.
Elsevier Science, Amsterdam, 1988.

[39] Oliver Prange, Tak Pan Wong, Kimberly Gerrow, Yu Tian Wang, and Alaa El-Husseini.
A balance between excitatory and inhibitory synapses is controlled by psd-95 and neuroligin.
Proceedings of the National Academy of Science, 101:13915–13920, 2004.

[40] Sylvia Rabacchi, Yannick Bailly, Nicole Delhaye-Bouchaud, and Jean Mariani.
Involvement of the n-methyl d-aspartate (nmda) receptor in synapse elimination during cerebellar development.
Science, 256:1823–1825, 1992.

[41] Indrani Rajan, Sonia Witte, and Hollis Cline.
Nmda receptor activity stabilizes presynaptic retinotectal axons and postsynaptic optic tectal cell dendrites in vivo.
Journal of Neuroscience, 38:357–368, 1998.

[42] Jose Ramos-Vera.
Technical aspects of immunohistochemistry.
Veterinary Pathology, 42:405–426, 2005.

[43] Laura Ritter, Alan Unis, and James Meador-Woodruff.
Ontogeny of ionotropic glutamate receptor expression in human fetal brain.
Developmental Brain Research, 127:123–133, 2001.

[44] Yildirim Sara, Thomas Biederer, Deniz Atasoy, Alexander Chubykin, Marine Mozhayeva, Thomas Sudhof, and Ege Kavalali.
Selective capability of syncam and neuroligin for functional synapse assembly.
Journal of Neuroscience, 25:260–270, 2005.

[45] Robert H. Scannevin and Richard L. Huganir.
Postsynaptic organization and regulation of excitatory synapses.

Nature Reviews: Neuroscience, 1:133–141, 2000.

[46] Peter Scheiffele, Jinhong Fan, Jenny Choih, Richard Fetter, and Tito Serafini.
Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons.
Cell, 101:657–669, 2000.

[47] Gerry Shaw.
A hek293 cell database.
http://www.mbi.ufl.edu/~shaw/293.html, March 2010.

[48] Gerry Shaw, Silas Morse, Miguel Ararat, and Frank Graham.
Preferential transformation of human neuronal cells by human adenoviruses and the origin of the hek 293 cells.
FASEB Journal, 16:869–871, 2002.

[49] Morgan Sheng.
Molecular organization of the postsynaptic specialization.
Proceedings of the National Academy of Sciences, 98(13):7058–7061, 2001.

[50] Ji-Ying Song, Konstantin Ichtchenko, Thomas Sudhof, and Nils Brose.
Neuroligin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses.
Proceedings of the National Academy of Science, 96:1100–1105, 1999.

[51] Yutaka Takumi, Vania Ramirez Leon, petter Laake, Eric Rinvik, and Ole Ottersen.
Different modes of expression of ampa and nmda receptors in hippocampal synapses.
Nature, 2:618–624, 1999.

[52] Harry Towbin, Theophil Staehelin, and Julian Gordon.
Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications.
Proceedings of the National Academy of Sciences, 76:4350–4354, 1979.

[53] Brigitte van Zundert, Akira Yoshii, and Martha Constantine-Paton.
Receptor compartmentalization and trafficking at glutamate synapses: a developmental proposal.
Trends in Neuroscience, 27:428–437, 2004.

[54] Frederique Varoqueaux, Gayane Aramuni, Randi Rawson, Ralf Mohrmann, Markus Missler, Kurt Gottmann, Weiqi Zhang, Thomas Sudhof, and Nils Brose.
Neuroligins determine synapse maturation and function.
Neuron, 51:741–754, 2006.

[55] K. Watabe, A. Ito, YI Koma, and Y Kitamura.
Igsf4: a new intercellular adhesion molecule that is called by three names, tslc1, sgigsf and syncam, by virtue of its diverse function.
Histology and Histopathology, 18:1321–1329, 2003.

[56] Nina Wittenmayer, Christoph Korber, Huisheng Liu, Thomas Kremer, Frederique Varoqueaux, Edwin Chapman, Nils Brose, Thomas Kuner, and Thomas Dresbach.
Postsynaptic neuroligin1 regulates presynaptic maturation.
Proceedings of the National Academy of Sciences, Early Edition:1–6, 2009.